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PREFACE

The high quality and thorough subject matter coverage of past volumes of the *Annual Review of Plant Physiology* testify to the hard work of their editors. Being new in this position, which I attained when Dr. Blinks was granted a sabbatical leave for a year at Cambridge, I can claim little honor for the present volume.

From my short experience, however, I have become fully aware of the work of the Editorial Committees that have labored on this and past volumes, and I wish to speak in their behalf. Each year this group meets and through a long and concentrated session it plans a future volume, selecting from a lengthy list of suggestions the particular reviews to be included and the authors to write them. To these men, whose names appear opposite the title page of each volume, should go credit for the wise and studied selection of topics for each review. In fact, in 1955, under the guidance of Dr. Blinks the Committee responsible for Volumes 8 and 9 spent two solid days organizing these volumes so that invitations to authors might, in the future, be extended some 20 months before the reviews are due. This lightens somewhat the load on the editorial assistants and gives the writers more time for covering their subjects.

This year at Storrs the Committee spent considerable time in reorganizing the subject matter in such a way that the maintenance of continuity of some of the major subdivisions might be made easier. Next year the same subject will probably be considered again, for in a field so dynamic as Plant Physiology, no set order can remain indefinitely.

From my experience on the staff of the *Annual Review of Plant Physiology*, I cannot speak too highly of the fine spirit of cooperation between the members of the Editorial Committees, the scores of willing scientists who have written reviews, and the many friends of the publication. For example, this year a scientist declined the invitation to review a certain subject because of previous commitments, but he named a colleague who was in a position to review a related topic. Another man, when solicited for a review for Volume 10, volunteered to write his paper for Volume 9 so that it could accompany a paper on a related subject and so make a more complete and unified coverage. Such is the spirit that has pervaded the atmosphere during the organization of Volume 10. It speaks well for the future of the review series.

The faithful service of Professor H. B. Tukey is gratefully acknowledged upon his retirement from the Editorial Committee on December 31, 1956. His successor is Dr. F. E. Gardner. The Editorial Committee has been enlarged by the addition of Dr. J. G. Horsfall who will represent the plant pathologists. It is a pleasure also to acknowledge the constant help of the present editorial assistant, Mrs. Sonya Pinney, and the always friendly advice of Dr. J. Murray Luck, managing editor of *Annual Reviews*.

A. S. CRAFTS

XUM

TOPICS AND AUTHORS
ANNUAL REVIEW OF PLANT PHYSIOLOGY
VOLUME 9 (1958)

MINERAL NUTRITION (GENERAL), *W. Reuther*
PHYSIOLOGY OF THE FRESH WATER ALGAE, *R. W. Krauss*
PLANT-SOIL RELATIONSHIPS (PHOSPHORUS), *L. A. Dean*
NITROGEN METABOLISM, INCLUDING PROTEINS, *E. W. Yemm*
QUANTUM YIELD OF PHOTOSYNTHESIS, *R. Emerson*
CYTOCHROME SYSTEMS OF PLANTS, *B. Chance and L. Smith*
THE NATURAL AUXINS AND INHIBITORS, *J. A. Bentley*
DESTRUCTION OF AUXIN, *P. M. Ray*
USES OF AUXINS IN FLOWER AND FRUIT CONTROL, *A. C. Leopold*
FLOWERING, *F. Lona*
HERBICIDES, *E. K. Woodford and K. Holly*
PHOTOTROPISM AND PHOTOTACTIC MOVEMENTS OF UNICELLULAR ALGAE, *J. Reinert*
TISSUE GROWTH FACTORS, *F. Skoog*
POST HARVEST PHYSIOLOGY OF FRUITS, *R. Ulrich*
THE TOBACCO PLANT, *R. A. Steinberg and T. C. Tso*
PHYSIOLOGY OF SALT TOLERANCE, *H. E. Hayward and L. Bernstein*
TRANSPIRATION, *W. T. Williams*
METABOLISM OF ASCORBIC ACID IN PLANTS, *L. W. Mapson*
PHYSIOLOGICAL ECOLOGY, *W. M. Hiesey*
BIOGENESIS OF PLANT PIGMENTS, *L. Bogorad*
MORPHOGENESIS IN LOWER PLANTS, *L. Jaffe*

CONTENTS

PREFATORY CHAPTER, *W. J. V. Osterhout* 1

APPARENT FREE SPACE, *G. E. Briggs and R. N. Robertson* 11

MINERAL NUTRITION OF PLANTS, *H. G. Gauch* 31

NITROGEN METABOLISM IN PLANTS: TEN YEARS IN RETROSPECT, *F. C. Steward and J. K. Pollard* 65

PHOTOCHEMISTRY OF CHLOROPHYLL, *J. L. Rosenberg* 115

BIOCHEMISTRY OF CHLOROPLASTS IN RELATION TO THE HILL REACTION, *K. A. Clendenning* 137

AUXIN RELATIONS IN ROOTS, *B. Åberg* 153

THE HISTORY AND PHYSIOLOGICAL ACTION OF THE GIBBERELLINS, *B. B. Stowe and T. Yamaki* 181

STOCK AND SCION RELATIONS, *W. S. Rogers and A. B. Beakbane* . . 217

ASCENT OF SAP, *K. N. H. Greenidge* 237

DROUGHT RESISTANCE IN PLANTS AND PHYSIOLOGICAL PROCESSES, *W. S. Iljin* 257

THE SUGARCANE PLANT, *G. O. Burr, C. E. Hartt, H. W. Brodie, T. Tanimoto, H. P. Kortschak, D. Takahashi, F. M. Ashton, and R. E. Coleman* 275

MASS CULTURE OF ALGAE, *H. Tamiya* 309

PERMEABILITY OF PLANT CELLS, *R. Collander* 335

PHYSIOLOGY OF PHLOEM, *K. Esau, H. B. Currier, and V. I. Cheadle* . 349

PHYSIOLOGICAL ECOLOGY, *W. D. Billings* 375

PHYSIOLOGICAL GENETICS, *H. J. Teas* 393

EFFECTS OF ANTIBIOTICS ON PLANTS, *P. W. Brian* 413

SOLUBLE OXIDASES AND THEIR FUNCTIONS, *W. D. Bonner, Jr.* . . . 427

INDEXES 453

XUM

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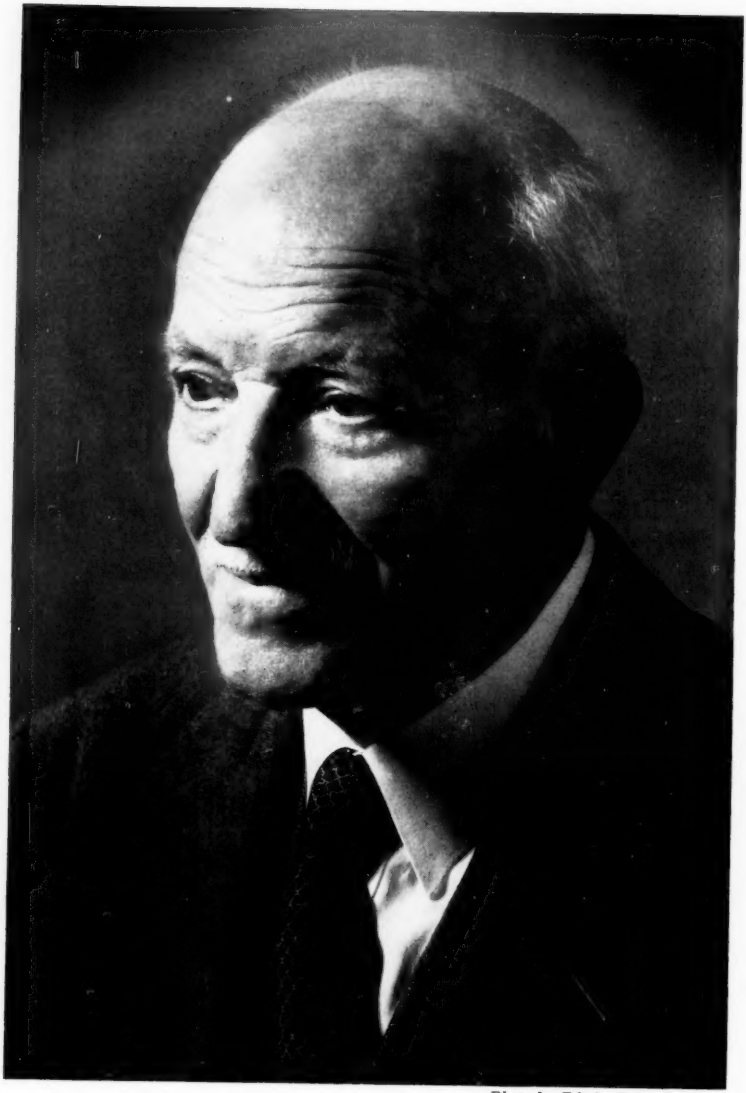
XUM



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W. J. V. OSTERHOUT

PREFATORY CHAPTER

THE USE OF AQUATIC PLANTS IN THE STUDY OF SOME FUNDAMENTAL PROBLEMS

By W. J. V. Osterhout

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The writer wishes to thank the editors of this series for the opportunity to present a brief summary (1) of some experiments carried out in his laboratory showing the value of certain aquatic plant cells in solving various fundamental problems. It is hoped that it may be of use to other workers.

The life processes of aquatic plants present problems of extreme interest which attracted the attention of the writer long before he had any formal instruction in this field. The first opportunity for investigation came in 1892 when as an undergraduate at Brown University he was invited to come to the Marine Biological Laboratory in Woods Hole, Massachusetts.

In studying the morphology of the red alga *Agardhiella tenera* (formerly known as *Rhabdonia tenera*) the writer observed a very interesting phenomenon. It was found that four spores, each of which could form a new plant, were able to combine and form a single plant (2). This is the converse of the experiment in which an egg in the four cell stage is shaken into four separate blastomeres and each develops into a complete embryo (3). In both cases the pattern of development laid down in the germ plasm had to be modified.

After joining the faculty of the University of California in 1896 the writer found that an interesting situation existed in the osmotic pressure of algae (4). Growing on steamers passing regularly from fresh water to sea water and back again at short intervals there was a flora consisting of red, brown, green, and blue-green algae. On arriving in salt water the cargo was unloaded shifting the water line and exposing the algae to the sun where the salt water quickly evaporated leaving the algae covered with salt crystals. A few hours later they were in fresh water after which they again returned to salt water. These severe changes in the osmotic pressure of the medium did not prevent the cells from growing. In view of this it became necessary to revise our ideas regarding the tolerance of living cells for extreme changes of osmotic pressure.

When Jacques Loeb came to the University of California bringing a lively interest in the antagonistic action of ions on animal cells it was natural to test these effects on plants (5). The results were striking. It soon became evident that the injurious effects of a toxic substance could be antagonized by another substance which was injurious when it alone was present. It was found that antagonism in plants follows the same rules as in animals so that monovalent cations are antagonized by bivalent and still more by trivalent cations. The effects of incomplete antagonism were studied and certain general considerations were formulated.

It was found that antagonism plays a role in permeability since antag-

XUM

onistic salts such as NaCl and $CaCl_2$ when present in the right proportions form a balanced solution which prevents the abnormal increase in permeability observed when only one salt is present (6). Attention was called to the relation between antagonism and Weber's law (7).

After going to Harvard in 1909 work on antagonism was continued when quantitative experiments on injury and recovery were carried out. For this purpose the marine alga *Laminaria* was used and determinations were made of the electrical resistance which depends chiefly on the concentration, electrical charges, and mobilities of ions in the nonaqueous surface layers of the protoplasm (8). These layers appear to act like guaiacol in taking up a great deal of water from the external solution.

Using solutions of the same resistance as sea water the following experiments were performed. It was found that on transferring the cells from sea water to pure NaCl the resistance decreased rapidly. This was reversible. In pure $CaCl_2$ the resistance of the cells increased but it returned to normal when the cells were replaced in sea water. La^{+++} acted like Ca^{++} but it was more effective.

It seems probable that the decrease in resistance caused by NaCl is due to an increase in the water content of the nonaqueous layers of the protoplasm. Shedlovsky & Uhlig (9) found that the resistance of dry guaiacol containing Na-guaiacolate decreased rapidly when water was added. This was due to increased dissociation of the Na-guaiacolate.

We may also assume that a decrease in water content is responsible for the increase in resistance in $CaCl_2$. This idea is supported by the experiment of Schmitt & Palmer (10). Using lipide extracted from the brain to imitate the nonaqueous surface layers of the protoplasm they found that when it was transferred from a solution of 0.6 *M* KCl to a solution of 0.04 *M* $CaCl_2$ the water in the lipide was promptly removed.

The reversible effects in NaCl and $CaCl_2$ on *Laminaria* were followed by irreversible effects which decreased resistance and which may have been due to changes (precipitation, coagulation, etc.) in the protoplasm on which the surface layers rest.

It therefore seemed that the resistance and the water content of the surface layers could be controlled by varying the proportion of Na^+ and Ca^{++} in the solution. Therefore it was later assumed (11) that the best proportion of Na^+ and Ca^{++} depends on the fact that it insures the most favorable water content. The permeability of the outer surface layer of the protoplasm appears to depend largely on the water content of this layer. According to Adolph (12) about two-thirds of the human body is water and an excess or deficit of only 1 per cent causes a noticeable disturbance.

It may be noted that Jacques Loeb (13) in discussing the antagonistic effects of Na^+ and Ca^{++} called attention to the fact that a sodium soap contains much more water than a calcium soap.

Somewhat similar results were obtained with organisms other than *Laminaria* including other marine algae and a flowering plant, *Zostera* (14).

A quantitative theory which enables us to predict the behavior of the cells in various mixtures can be constructed by assuming that the resistance

is proportional to a substance M having a high resistance (and containing very little water) which is produced in the nonaqueous surface layers. We may assume that this substance results from the chain reaction $A \rightarrow M \rightarrow B$ in which the reaction $A \rightarrow M$ is catalyzed by Ca^{++} and the reaction $M \rightarrow B$ is catalyzed by Na^{+}. Other monovalent cations act like Na^{+} and bivalent cations act like Ca^{++}.

When H^{+} was added the resistance of *Laminaria* rose and then fell as the concentration of acid gradually increased by diffusing into the cells. This suggested that amphoteric substances in the cells acted like gelatin in the experiments of Jacques Loeb (15) in which as the acid increased the water content fell until the isoelectric point was reached. It then rose as more acid was added after the isoelectric point was passed.

The spread of injury presents very interesting features. When a cell of the marine alga *Griffithsia* (16) is injured at one end by pressure, the small chromatophores at that end give up their red coloring matter which diffuses inside the cell. When it comes in contact with the next chromatophores, they give up their coloring matter in turn so that a wave of injury passes along the cell and death soon follows.

When one end of a *Nitella* cell is cut a "death wave" spreads rapidly along the cell killing the protoplasm as it progresses (17).

If injury to a cell produces a toxic substance, it may diffuse to other parts of the cell (18). When a colorless cell of the Indian Pipe (*Monotropa*) is cut, the injured cells become black. The darkening begins at the nucleus and spreads to other parts of the cell.

It may be noted that some organisms show a surprising ability to survive injurious influences. This was seen very clearly in an experiment with a branch of *Tradescantia* (19). A small branch without any root was placed on a dry table top before a north window and left to its own devices without receiving any water. In two years it increased in length almost 150 per cent although losing water constantly. This growth is attributable to the fact that new cells are formed which are able to take water from the older cells, at the same time they absorb CO_2 from the air and synthesize carbohydrates during exposure to light.

When cells of *Ulva* are transferred from darkness to light the rate of photosynthesis gradually increases (20). This indicates that a substance which promotes photosynthesis disappears in darkness and is restored in light (photoinduction).

It became increasingly evident that to find out what goes on inside the cell we need large cells whose sap can be obtained without contamination and on which electrical measurements can be made by applying electrodes at two or more points on the surface or by inserting electrodes into the cell. Improvements in technique at this time made it possible to measure very minute electrical current. Fortunately in 1920 the writer found the fresh water plant, *Nitella flexilis*, with cells several centimeters in length. The writer took advantage of this at once and the work was subsequently aided by a grant from the Carnegie Institution of Washington.

Two large marine cells, *Valonia macropysa* and *Halicystis Osterhoutii*,

XUM

were later studied in Bermuda with the aid of a grant from the Rockefeller Institute for Medical Research. These species studied in the writer's laboratories will be referred to in the future simply as *Nitella*, *Valonia*, and *Halicystis*.

In 1925 the writer joined the staff of the Rockefeller Institute for Medical Research where it was possible to make extensive studies of these cells in Bermuda and in New York.

At various intervals the writer was assisted in Bermuda by W. C. Cooper, Jr., M. J. Dorcas, E. B. Damon, and A. G. Jacques and in New York by E. S. Harris, Jr., S. E. Hill, S. E. Kamerling, J. W. Murray, and W. M. Stanley. L. R. Blinks and M. Irwin worked independently in the writer's laboratories in Bermuda and in New York.

These cells afforded an excellent opportunity to study the composition of the sap, the osmotic effects, the permeability, and electrical phenomena.

In all these cells the protoplasm forms a thin layer (a few microns thick) surrounding a large central vacuole filled with sap. The protoplasm contains numerous ellipsoidal chlorophyll bodies and is presumably multinucleate. Outside the protoplasm is a cellulose wall which is very permeable to electrolytes.

In Bermuda a very interesting situation was found. *Valonia* (21) and *Halicystis* (22) with cells each having a few milliliters of sap were found to differ greatly in their power to absorb electrolytes from the sea water. At that time many physiologists believed that the electrolytes in the vacuole were largely in combination with organic matter and that considerable metabolism occurred in the vacuole. But in these cells very little organic matter was found inside the vacuole although the concentration of electrolytes in the sap was about 0.6 *M*. It thus appears evident that metabolism involving organic substances is carried on almost entirely by the protoplasm which may occupy less than 1 per cent of the volume of the cell.

In *Valonia* (21) the sap contains about 0.5 *M* KCl which is about 40 times the concentration of KCl found in the sea water. But the internal concentration of NaCl is much less than that of the sea water, and very little $MgSO_4$ is taken up (21).

In *Halicystis* (22) the composition of the sap is not very different from that of the sea water except that, as in *Valonia* (21), very little SO_4 enters the cell. The pH value of the sap is between 5 and 6 as in *Valonia* (22) and that of the sea water is about 8.2. This *Halicystis* previously known as *Valonia ventricosa* when the sap was first analyzed (22) was later renamed *Halicystis Osterhoutii*, Blinks & Blinks (23), and the sap was subsequently analyzed by Blinks & Jacques (24) who confirmed the previous analysis (22). According to Cooper & Blinks (25) the sap of *Valonia ventricosa* collected in Florida and properly identified was found to have about the same composition as the sap of *Valonia macrophysa* (21) found in Bermuda.

The accumulation of certain ions inside the cell may be very great. In the sap of *Halicystis* there is at least 500 times more nitrate than in sea water. In the sap of *Valonia* there is at least 2000 times more nitrate than in sea water (26).

The difference in the sap of these two cells produces a striking difference in their behavior (22). *Halicystis* at first grows attached to the coral rock below the surface of the sea water but later becomes detached and rises to the surface and floats freely. *Valonia* also grows attached but when set free it does not float but it can be made to do so by adding to the sea water NH_4Cl which enters the sap and lessens its specific gravity without apparent injury to the cells (27). This has a bearing on the problem of the behavior of plankton which may float or sink under different conditions.

The sap of the fresh water plant, *Nitella*, presents an interesting contrast to what we find in *Valonia* and *Halicystis*. According to Irwin (28) the sap contains about 0.13 *M* halide and the external solution has a very small amount of electrolytes. We find in the sap about 0.05 *M* KCl and 0.05 *M* NaCl (22, 29). The sap gives a xantho protein test (30). Most of the electrolytes are in ionic form (29). The pH value of the sap is about 5.5 and the external pH value is about 6.5 (30, 31). The sap shows some buffer action. It is interesting to note that the pH value of the sap in all of these cells is between 5 and 6 in spite of the fact that the pH value of the sea water is much higher than that of the fresh water.

From time to time analyses were made of the sap of *Nitella* used in the writer's laboratory. Attention should be called to the classic work of Hoagland and his associates (32, 33) and that of Collander (34) on the composition of the sap of various species of *Nitella*.

Cells of *Nitella* in pond water have an internal osmotic pressure of about 6 atm. The osmotic pressure of *Valonia* and *Halicystis* is slightly higher than that of the sea water.

Interesting results are obtained when we measure the flow of water through the *Nitella* cell.

If a cell of *Nitella* is divided into two parts, A and B, by a ring of vaseline and water is placed on one end at A and sucrose solution is placed on the other end at B, the water enters at A and passes along inside the cell and escapes at B (35). During this process particles and solutes move from A to B and their concentration decreases at A and increases at B. This is strikingly shown when the vacuole contains brilliant cresyl blue; the blue color moves from A to B so that the color becomes paler at A and deeper at B (35). Most of the dye is in the form of ions. Decrease in the concentration of electrolytes at A decreases the potential at A and increase in the concentration of electrolytes at B increases the potential at B. When the sucrose at B is replaced by water the flow of electrolytes is reversed and the original potentials are restored at A and B (36).

Contraction of chloroplasts occurs at A when water enters the cell at A and passes along inside the cell to B and escapes at B into the external sucrose solution. When this is reversed by replacing the sucrose with water at B the chloroplasts at A expand (35). If injury occurs the chloroplasts become displaced and disorganized. At B the adjacent rows of chloroplasts separate at certain places producing clear areas known as splits (37). Some of these effects may be connected with the precipitation of globulins and similar substances. The contraction of chloroplasts may be inhibited by adding

XUM

dilute mammalian blood or duponol to the entering stream of water (35).

An apparatus was devised to measure the movement of water in cells of *Nitella* (38). A small portion of the cell is surrounded by a cork. One end of the cork is fitted to a glass tube drawn out to a calibrated capillary and the other end of the cork is fitted to another glass tube without a capillary. Both tubes are at first filled with water. The water in the latter tube is now replaced by a solution and the movement of water in the cell is measured in the capillary tube. By knowing the internal and external osmotic pressures and the volumes of the solutions at both ends of the cell it is possible to predict the total amount of flow (38). Transport of water from concentrated to dilute solutions inside the cell can also be measured and calculations made on this basis (38). This may help to explain the problems of excretion of water against a gradient in other cells. If the osmotic pressures are different at opposite ends of the root hair water may be forced into the root and cause a rise of sap.

A substance can enter a living cell and reach a higher concentration inside than outside. This requires energy which is produced by metabolism. When a cell of *Nitella* containing 0.05 *M* KCl is placed in 0.001 *M* KCl solution we find that KCl enters and maintains the concentration of 0.05 *M* KCl as the cell grows. It thus enters against the gradient. To account for this we may assume that K^+ and Cl^- combine at the outer surface of the protoplasm with organic molecules (39). These diffuse inward where they are decomposed by metabolism so that K^+ and Cl^- appear as free ions.

In accordance with this it was found that when the outside concentration of NH_4Cl increases (40, 41) the rate of entrance into the vacuole of *Valonia* does not increase in proportion because there are not enough carrier molecules to make this possible.

To illustrate the behavior of carrier molecules we may consider an artificial cell (42) in which the nonaqueous layer is represented by a mixture consisting chiefly of guaiacol which can take up a good deal of water without entirely dissolving in it.

Outside of this layer is an aqueous solution of KOH + KCl and inside the layer a solution of CO_2, representing the protoplasm in which CO_2 is constantly produced. It is then found that K^+ unites with the guaiacol to form K-guaiacolate which passes through the guaiacol and reacts with the CO_2 in the artificial protoplasm to form K_2CO_3 and $KHCO_3$. The concentration of K^+ in the artificial protoplasm becomes much higher than in the external solution because K^+ cannot pass out through the guaiacol layer except very slowly. This produces an outwardly directed potential which is presumably due to the fact that K^+ has a higher mobility in the guaiacol layer than the anions with which it is associated. This imitates certain processes in the living cell of *Nitella*.

Electrical measurements on *Nitella* indicate that the carrier molecules do not give up all of their ions until they reach the vacuole. Hence it may happen that if the protoplasm has the same concentration of potassium compounds as the vacuole, the dissociation of these compounds in the proto-

plasm may be less than in the vacuole and consequently the outwardly directed potential across the inner surface of the protoplasm may be greater than the outwardly directed potential across the nonaqueous layer of the living protoplasm in contact with the external solution.

The guaiacol molecule may be regarded as a carrier molecule which picks up K^+ from the external solution and delivers it to the artificial protoplasm.

The K-guaiacolate is only slightly dissociated in the guaiacol layer because the dielectric constant is relatively low, so that in this respect the guaiacol layer would resemble the nonaqueous surface layers of certain living cells.

We may assume that in the nonaqueous layer of the living cell there are amphoteric compounds and other substances which carry Cl^- and other anions into the protoplasm.

In the artificial cell (43) the concentration of KCl may become greater in the artificial protoplasm than in the external solution thus setting up an outwardly directed potential but in spite of this ions continue to enter in combination with carrier molecules just as in the living cell of *Nitella*.

In addition to the entrance of ions in combination with carrier molecules there may be some inward and outward passage of free ions (44) as shown by the existence of inwardly and outwardly directed protoplasmic potentials.

Let us now consider the behavior of ions in the outer surface layer of the protoplasm. This layer is nonaqueous but may contain considerable water and is very permeable to water. In *Nitella* it may be much more permeable to water than to ethyl alcohol (45).

The nonaqueous surface layer may be invisible under the microscope and is then regarded as less than half a micron in thickness.

Weak electrolytes enter the cell much more rapidly in the form of molecules than in the form of ions. This is clearly seen in the experiments of Irwin (46). The undissociated form of brilliant cresyl blue enters the vacuole of a *Nitella* cell, in which it is dissociated and rapidly accumulates. The rate of penetration of the dye follows the course of a reversible pseudounimolecular reaction with a high temperature coefficient, involving expenditure of energy derived from the activity of the cell.

H_2S and CO_2 also penetrate in the form of undissociated molecules into *Valonia* (47, 48).

Overton (49) found that various organic substances enter the cell at different rates which correspond roughly to their solubility in such substances as olive oil. He therefore concluded that the protoplasmic surface is lipoid in nature. This, however, makes no provision for the entrance of salts or for the free passage of water.

Overton (49) made experiments by plasmolyzing cells with solutions of salts. He stated that on standing in such solutions the contracted protoplasm did not subsequently expand as it should if the salts entered. The writer (50) repeated these experiments and found that the expansion actually occurred, showing that the salts did enter. This was overlooked by Overton because

XUM

when the cells remained in the salt solution they subsequently died and the protoplasm shrank, producing false plasmolysis which could easily be mistaken for true plasmolysis.

Let us consider further the electrical properties of these cells. It is evident that ions can enter in combination with carrier molecules without producing any inwardly directed potential and this is what we usually find in *Nitella*. We can therefore understand why the concentration of ions inside the cell increases while the potential remains outwardly directed.

Let us now lead off by means of a string wet with 0.001 *M* KCl from a spot (A) on a *Nitella* cell (suspended in air) with an outwardly directed potential of 97 mv. to a spot (B) where the potential is zero. An exceedingly minute current flows through the measuring instrument to (B). This is sufficient to register in a string galvanometer attached to an amplifier (51, 52). Blinks states that under certain conditions a current amounting to 1 to 5 μ amp. is found to flow in the marine alga *Halicystis* in sea water where there is an outwardly directed potential of 70 mv. (53).

In order to measure the potential in *Nitella*, a spot, B, at one end of the cell is killed with chloroform (51). From another spot, A, we lead off to B and measure the potential. As long as this potential remains constant we conclude that no injury has spread from B to A and consequently measurements made between B and points beyond A give correct values. For each lot of cells we ascertain what concentration of KCl gives zero potential and use this concentration instead of chloroform for a reference point (52, 54). In this case no injury to any part of the cell occurs.

It seems possible that we may have an ingoing stream of ions combined with carrier molecules producing no potential while entering and in addition a smaller outgoing stream of free ions producing a considerable potential.

It may be noted that if the external concentration of KCl applied to *Nitella* is sufficiently high (e.g., 0.1 *M* KCl) there is an inwardly directed potential which means that some K^+ enters in the form of free ions (55). At the same time an additional amount of K^+ may enter in combination with carrier molecules producing no potential while entering.

When we plot the logarithm of the concentration of applied KCl against potential we obtain an approximately straight line having nearly the slope demanded by the Nernst equation for diffusion potential (52, 56). It thus appears that the protoplasm behaves somewhat like a potassium electrode.

If we consider a series of points on a *Nitella* cell leading from left to right, A, B, C, D, E, F, etc., with an outwardly directed potential at each point we can stimulate the cell by reducing the potential at A to a low value so that B discharges into A; C then discharges into B and thus a progressive electrical disturbance passes along the cell, as each spot discharges in turn. If a spot D is unable to discharge we might expect that when we lead off from a spot E by means of a salt bridge, consisting of a string moistened with 0.001 *M* KCl , to C, then E will be able to discharge into C, after which F can discharge into E so that an electrical disturbance can progress along the cell. The experiment shows that this actually takes place thus proving that our picture of the process is correct (57). The experiment, therefore, has an important theoretical significance.

Mention of other researches must be omitted here for lack of space but it is hoped that enough has been said to indicate the advantages of large plant cells for the study of certain important problems and to outline some simple methods of procedure.

Others have contributed to our knowledge of these cells. Lawrence R. Blinks has been working for many years in this field and has made many important contributions. The writer wishes to thank those who have helped him in his experiments.

It may be added that in order to understand the physiology of the cell we need the aid of taxonomy, morphology, and the exact sciences. Thorough familiarity with the behavior of the living material is highly important.

These and other experiments on large plant cells show that the living cell has a remarkable power to select ions from the environment. These ions may accumulate in the interior of the cell until its osmotic pressure is higher than that of the external solution. This depends on the properties of the nonaqueous layers of the protoplasm and on conditions within the cell. This involves expenditure of energy.

The presence of these ions may produce potentials and as a result the cell may be capable of electrical stimulation in a manner resembling that of mammalian nerve.

This picture of the plant cell shows that it has many remarkable properties and suggests a wealth of problems for future investigations.

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APPARENT FREE SPACE[1]

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The difficult problems associated with the entry of dissolved substances into plant cells have been made more difficult by failure to envisage the properties which must be attributed to the cells in order to account for their known behaviour in a wide range of treatments. It is generally accepted that there is, between the vacuole and the outside of a plant cell, a barrier or barriers which offer a high resistance to the passage of many solutes including salts of strong acids and bases. The tonoplast which separates the vacuole from the cytoplasm is accepted as one such barrier, but many assume that there is also another barrier at the outside of the cytoplasm adjoining the cell wall. Although we believe that no such barrier exists for many solutes we do not propose to consider all the evidence in detail, nor is it the purpose of this article to survey the history of what we believe to be erroneous concepts. It might, however, be pointed out that while some investigators [e.g., Arisz (1)] have not subscribed to the widely accepted view, others [e.g., Lundegårdh (15)] have described the surface of the root cell as a membrane of oriented lipoid molecules adjoining the cell wall and resistant to the entry of dissolved substances. Recently Lundegårdh (16) has come to the conclusion that the evidence for a free space necessitates the abandonment of this earlier hypothesis.

Little has been published on the concept of apparent free space and nothing has been published on the complementary concept of apparent osmotic volume, terms which have long been in use in Cambridge. Since there has been some misunderstanding of these terms and their significance, it is desirable that they should be examined in some detail. Since only the outlines can be given in an article of this type, a more detailed treatment of the concept setting out the basis for many of the estimations given in this paper will be published elsewhere [Briggs (4)]. In 1948, Briggs & Robertson (6) pointed out that the tissue in which the intercellular spaces have been filled with water behaves as if the movement of larger molecules (e.g., sugar) and of electrolytes were restricted mainly to the space around the vacuoles; this space was discussed as the phase in the tissue in which solutes move relatively freely and from which they are removed into the vacuoles. This space, the "free space," cannot be measured unless its properties relative to the measuring system are known. We can therefore get only estimates of its dimensions. For these estimates, the term "apparent free space" was proposed by

[1] The survey of literature pertaining to this review was concluded in June, 1956.

XUM

one of us (G.E.B.) and used to interpret particularly the changes which occur in plant cells immediately after they are changed from one concentration of solute to another. Prior to any publication of the meaning of the term apparent free space, Hope & Stevens (12) sought permission to use the term, with due acknowledgements, in interpreting their results. After the term appeared in their paper, it was used by Butler (7) in an interpretation of experiments on wheat roots and has been discussed by Hylmö (13), Hope (11) and Epstein (8). Apart from these references, the literature contains little direct discussion of apparent free space, though some of the published data on the uptake of various substances can be reinterpreted using this concept. This article will therefore attempt to review, in general terms, the evidence for the free space and refer to some of the earlier published work on solute uptake, particularly of electrolytes.

The complementary concept to free space—"osmotic volume," the region of the cell or tissue which is not rapidly penetrated by the solute and only by the solvent—is necessary to explain the plasmolytic behaviour of cells. A term which clearly might be appropriate for this region would be osmotic space but since for a long time at Cambridge the term "osmotic volume" has been in constant use although not having appeared in any published paper, we propose to continue it. As with free space, only estimates of osmotic volume based on assumed properties of the system can be obtained. These estimates are termed "apparent osmotic volume."

The concepts of free space, apparent free space, osmotic volume and apparent osmotic volume will first be defined. The methods of determining the apparent free space and the effect of the neglect of properties of the system on the estimates will then be discussed. The location of the free space in the tissue will be examined and its relevance to the interpretation of ion exchange, electric potential differences, swelling of the cytoplasm, and times of adjustment of tissues to changes of external solution. Some of the published results obtained by other investigators are reinterpreted on this basis and the implications in relation to the uptake of substances such as metabolites commonly used experimentally are discussed. Finally there are many suggestions as to how these considerations help in a better understanding of the physical properties of the cytoplasm generally.

Definitions

Initial uptake.—When a cell or a tissue is transferred from a dilute to a more concentrated solution, there is a period of rapid uptake of salt, followed by a period of less rapid uptake. This early absorption has been called the initial uptake. For the purpose of analysis it is assumed that during this period both accumulatory uptake, probably mainly into the vacuole, and uptake into the free space, which we shall call free-space uptake, are taking place and that the latter is completed during this early stage. Some writers [Epstein & Leggett (9)] use the terms "active transport" and "exchange adsorption" for these two uptakes when considering uptake of the cations of a salt.

For electrolytes the initial uptake is frequently the same whether measured as the uptake of an anion from the external solution (accompanied by an equivalent amount of cation) or measured by the net change in external conductivity. The amount of a cation taken up during the initial uptake period is usually in excess of the amount of anion taken up simultaneously, because of the exchange of the cation from the external solution with the cations present in the free space of the tissue. Since, during the period of initial uptake, accumulation is taking place also, some allowance for this must be made to obtain the free space uptake (F.S.U.) during this period. The difficulties of allowing for the accumulation will be discussed later, but it is this free-space uptake that is used in the calculation of apparent free space. There has been some confusion in the literature due to failing to distinguish between initial uptake and free-space uptake.

Apparent free space and apparent osmotic volume.—The free space (F.S.) of a cell or tissue is that part into which the solute and solvent from the external solution penetrate readily; this is in contrast with that part of the cell or tissue into which the solvent but not the solute penetrates readily—the osmotic volume (O.V.).

As pointed out by Briggs & Robertson (6), in discs of plant tissue with the intercellular spaces injected with water the resistance offered to the passage of CO_2 is not very different from that offered by a layer of water of equal thickness, but for larger molecules, such as sucrose and for electrolytes, the discs behave as though the movement of these substances were restricted to a small fraction of the path; Briggs & Robertson showed that the coefficient of diffusion of potassium chloride in carrot tissue was only one-fiftieth of the coefficient of diffusion of that salt in water, indicating that the path in the tissue (which itself is about 86 per cent water) is very much restricted. Simultaneous measurement of the potential difference between solutions of different concentration on the two sides of the tissue showed that the potential difference was that which might be expected if the medium through which the potassium chloride passed contained an electrolyte the anions of which were nonmobile. This suggested that some of the free space of the tissue is in the cytoplasm. Experimental evidence obtained by Arisz (2) for leaves of *Vallisneria*, with no solution in the intercellular spaces, also indicated that the entry and movement of electrolytes and sugars in the cytoplasm was much more rapid than entry into the vacuoles.

Free space, however, cannot be estimated directly and this difficulty led to the introduction of the concept of apparent free space (A.F.S.). In deciding upon a useful definition of A.F.S. many difficulties which cannot be discussed here are encountered. We shall adopt the following definition: *If U is the estimated amount of the free-space uptake of a solute by tissue originally devoid of this solute from a solution of concentration C, then A.F.S. is U/C.* This, the apparent volume of the free space, is therefore the volume of the free space if at equilibrium its concentration of the solute were the same as that of the external solution. The amount readily entering the free

XUM

space is not necessarily the same as that readily leaving when the tissue is returned to a large bulk of water since some which entered may have become bound. The meaning of U is clear for substances which do not undergo ionisation but requires further consideration, as we shall show, for electrolytes. Hence, from our definition, if the free space were occupied by water containing nothing that reacted in any way with the solute, the volume of the free space would be equal to that of A.F.S. With a solute such as carbon dioxide the A.F.S. approximates to the volume of the water in the tissue. Of course there may be some of the water not available to the carbon dioxide and there may be some substances in the water which combine with the carbon dioxide. With a solute such as an electrolyte, probably the only part of the free space which is free of substances that affect the concentration of the electrolyte, is the water in the intercellular spaces as distinct from that in the wall and the cytoplasm. The amount of solute entering the free space and hence the size of the A.F.S. depends not only upon the size but also upon the properties of the free space and what it contains relative to the solute. We have separated the rapid uptake into a quick equilibration with the free space and a slower accumulatory uptake, but more sensitive methods suggest that the former component is probably not simple but consists of at least two processes—one more rapid and the other less rapid. Hence a knowledge of the method of estimating U is important.

Methods of Determining Apparent Osmotic Volume and Apparent Free Space

The weighing method.—The apparent osmotic volume of tissues such as discs, cut thin to facilitate equilibration, can be measured by weighing experiments. From the weight of the discs in water (W_w), and then in solution (W_s), and finally in air (W_a) (with appropriate extrapolation of the curves for changing weight against time to allow for slower drifts due to respiration, accumulation, etc.), the final volume of the whole tissue and of that not apparently penetrated rapidly by the solute i.e., A.O.V., can be calculated. The final volume of the tissue in solution is $W_a - W_s/\Delta_s$ and the A.O.V. is $W_w - W_s/\Delta_s - \Delta_w$ where Δ_s is the density of the solution and Δ_w is that of water. The difference between the total volume (V) and that not apparently penetrated by the solute (A.O.V.) is the apparent free space (A.F.S.), since in the calculation the osmotic volume is assumed to be surrounded by a medium of the same concentration as the external solution. A more detailed account of this method will be published elsewhere (Briggs, Blyth, and Mercer).

The A.O.V. decreases with increase of the osmotic pressure (P) of the external solution. Table I shows data (obtained by Blyth, but typical of many collected at Cambridge) for carrot discs in KNO_3 solution with no air in the intercellular spaces. If A.O.V. is plotted against the reciprocal of the osmotic pressure, P, it can be seen that above 10 atm. ($1/P < 0.1$) the tissue behaves like an osmometer containing a cane sugar solution where $P(V-b) = K$, i.e.,

TABLE I

RELATION BETWEEN THE OSMOTIC PRESSURE OF THE EXTERNAL SOLUTION AND THE A.F.S. AND A.O.V. COMPARED WITH ORIGINAL VOLUME OF TISSUE IN WATER

Concentration	$\frac{1}{P.}$	$\frac{\text{A.F.S.}}{\text{original}}$	$\frac{\text{A.O.V.}}{\text{original}}$
0.08 *M* KNO_3	0.293	0.09	0.95
0.16 *M* KNO_3	0.149	0.135	0.845
0.28 *M* KNO_3	0.088	0.235	0.68
0.40 *M* KNO_3	0.064	0.30	0.525
0.70 *M* KNO_3	0.038	0.425	0.364

A.O.V. rises linearly with 1/P. This represents the range where the cells have lost turgor. In this range, the apparent free space increases rapidly with increasing concentration.

At lower osmotic pressures ($1/P > 0.1$) where the cells are not plasmolysed, the A.F.S., i.e., the total volume less A.O.V., increases from less that 10 per cent at 3 atm. to about 20 per cent at 10 atm. This change will be referred to later. Apart from the change of the A.F.S. with concentration, the nature of the solute used in the weighing method (as in all methods) may affect the magnitude of A.O.V. and A.F.S. Thus the A.O.V. of carrot discs determined in sugar solution which causes plasmolysis is about 10 per cent higher than that determined with salt solutions of the same osmotic pressure. This is not due to a different effect on metabolism but can reasonably be attributed to a difference in the amounts of the different solutes adsorbed or bound in parts of the cytoplasm. Similar results were obtained with gelatine gels.

In summary, the weighing method can be used to determine A.O.V. and A.F.S. for a particular solute at a particular concentration.

Methods dependent on uptake of solute.—(*a*) Entry of nonelectrolytes. The simplest method of determining A.F.S. is to use solutions of a nonelectrolyte which is not already present in the tissue. Preferably it should be one which is not accumulated so that there is no need to make allowance for accumulation.

Despite the simplicity of this method, little use seems to have been made of it. Butler (7) used essentially this method to determine A.F.S. of wheat roots with mannitol as the solute, though in his technique, he allowed the roots to equilibrate with mannitol and then estimated the amount of mannitol (U) in the free space by allowing it to diffuse out of the roots into an external solution containing no mannitol. Since Butler found no significant differences in A.F.S. whether calculated from data for 1 hr. or 2 to 3 hr. periods, there was no slow process releasing mannitol. Further, the fact that the A.F.S. for roots which had been in contact with 20 m*M* mannitol was not different from that of roots which had been in contact with 10 m*M* mannitol

XUM

suggests that the amount adsorbed is probably negligible—a constant fraction is unlikely.

(*b*) Entry of electrolytes. This method is essentially the same as that for the entry of nonelectrolytes except that many salts are accumulated and allowance must be made to obtain free-space uptake (F.S.U.). If the free space contains no ions other than those free to pass out into the external solution, then there is no difficulty in attributing a meaning to U, the amount of salt not already present entering the free space. The usual assumption is that accumulation has proceeded at a steady rate from zero time and that the extrapolation to zero time of the line for amount accumulated gives F.S.U. (see Fig. 1). This extrapolation will underestimate U because the ac-

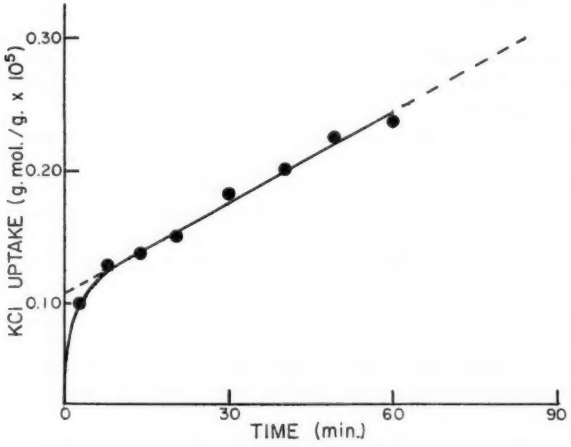

FIG. 1. Relation between KCl uptake and time with extrapolation to give free space uptake.

cumulation rate presumably rises with time from zero in a way which at present is unknown. A.F.S. can therefore be determined more accurately where the accumulation rate is relatively low as it is in the presence of cyanide or other inhibitors, or at low temperatures or, for some tissues, in a salt with a divalent anion.

As with nonelectrolytes, a combination of a salt with substances in the free space tends to make the A.F.S. greater than the F.S. The presence of nonmobile ions in the free space introduces further complications. If, for example, the free space contains nonmobile anions (in concentration A), as many plant tissues appear to do, then the mobile ions present will be unequal in concentration, but the product of their concentrations (if they are both univalent) will be equal to the product of the concentrations in the external

solution with which the free space is in equilibrium. If the concentration of the solution with which the free space was initially in equilibrium is very small compared with that of the solution used to measure A.F.S., then we can neglect the very small concentration of mobile anions originally in the free space, and need consider only the mobile cation, *k*, which will have a concentration equivalent to A. If the tissue is now placed in a solution of a salt such as KCl, then if the *k* ions in the free space are potassium, the amount of K and Cl entering will be the same. If *k* is some other univalent cation, the amount of Cl ion entering will not be affected but more K will enter in exchange for *k*. We have adopted the procedure of taking the amount of anion entering to estimate A.F.S. If the amount of cation were used, the A.F.S. would be greater because of exchange and might exceed the free space. Epstein & Leggett (9) have published results with Sr^{++} entering at 1.5×10^{-3} equiv. per kg. from a solution of 1.0×10^{-3} equiv./l. If we used these figures we should calculate 1500 ml. A.F.S./kg. As we shall see later, if *k* is bivalent, then for the same concentrations expressed in equivalents, more Cl enters than when *k* is univalent.

If the free space had been in equilibrium with a solution whose concentration was not negligible, then the concentration of mobile anions in the free space would, if different, exchange with those in the measuring solution or, if the same, reduce the amount entering; this raises difficulties which are best avoided by ensuring that the free space is initially in equilibrium with a solution of negligible concentration before attempting to determine A.F.S.

If the free space contained several phases, some with nonmobile cations, as well as some with nonmobile anions, there would be further difficulties; these cannot be considered here but the possibility of such complexity must be borne in mind when considering the significance of A.F.S. where the amount of entering anion is used as a measure of *U*.

As already pointed out, the A.O.V. of carrot discs indicated by sugar solution is greater than that indicated by isosmotic salt solutions (e.g., KCl); this difference can be explained by a combination of the salt with some substances in the free space. Evidence for such a combination of KBr is provided by the results of experiments by Sutcliffe (21) with beetroot discs, if the material in his two sets of experiments is comparable. From initial uptake, the amount of K^+ entering the free space from KBr of concentration 20×10^{-3} *M* is about 15.5×10^{-3} moles/kg. and that of Br about 5×10^{-3} moles/kg., while in another experiment, the amount of readily exchangeable K^+ is only 10.5×10^{-3} moles/kg. and of Br^- only 1.5×10^{-3}, the differences between these amounts suggesting a component of the initial uptake amounting to 3 to 5×10^{-3} moles/kg. which is fixed in some way as not to be readily exchangeable. Further evidence for the view that with some salts there is uptake of this type in addition to ions going into a simple Donnan system or in free water is found in the results of experiments recorded by Stiles & Skelding (20). Their results with $MnCl_2$ are simplest to interpret. If there is a non-

XUM

mobile anion in the Donnan system, the ratio of mobile anions inside to outside should rise with external concentration, while for free space with no nonmobile anion, the ratio should be constant. In one of their experiments, the percentage of chloride estimated to have entered the free space falls from 29 per cent to 10 per cent to 9 per cent of the external as its concentration rises from 0.2×10^{-3} to 1.0×10^{-3} to 5×10^{-3} M; for another experiment the percentages are 35 per cent, 19 per cent and 8 per cent. Stiles & Skelding recognize that this drift in the percentage is in the wrong direction for a Donnan system and, referring to the account given by Briggs & Petrie (5) of multiphase Donnan systems, suggest that the greater percentage for the dilute solution might be explained by some of the chloride ions having gone into the vacuole. Our estimates, unlike the figures used by Stiles & Skelding, are for the free-space uptake and therefore allow for the accumulation of chloride in the vacuole. We are inclined to attribute the difference to a fixation of a non-Donnan type which takes place relatively rapidly. These bound ions are presumed to exchange relatively slowly.

If the assumption is made that the uptake of salt can be measured by change in conductivity of the external solution, estimations of A.F.S. are subject to error through neglect of the exchange of ions in the solutions with ions of different mobility in the tissue. Fall in external conductivity can be used to estimate free space only if (*a*) exchange is negligible compared with the uptake of salt, as in high external concentrations, or (*b*) the exchange occurs between ions of approximately the same mobility.

For various reasons then the estimate of A.F.S. from entry of electrolytes will depart from the true free space. Some idea of the magnitude of the departure under different conditions may be obtained from the following examples which deal with a hypothetical system containing only a Donnan system. The ratio A.F.S./F.S. of such a system changes with changing concentration salt in the external solution. This is illustrated by Figure 2 where the ratio A.F.S./F.S. is plotted against the concentration, a_0, of the solution in which the system was placed. If the Donnan system containing A balanced by a univalent mobile cation were placed in a uni-univalent salt such as KCl and A.F.S. determined from the amount of Cl ion entering, or from the amount of KCl entering where the univalent ion balancing A is K, then the ratio A.F.S./F.S. rises with external concentration as shown in Curve 1. If the cation balancing A were divalent and A.F.S. were determined from Cl ion entering, then A.F.S./F.S. would follow Curve 2. If, however, conductivity were used as the measure of Cl ion entering and the mobilities of the balancing cations were not the same as that of K, A.F.S./F.S. would be quite different and curves 3, 4 and 5 are those to be expected if A were balanced by Na^+, Mg^{++} and Ca^{++} respectively; A.F.S./F.S. would increase markedly with decreasing concentration in the external solution and would eventually exceed unity. The volume of the solution is 60 times that of the Donnan system.

If the A.F.S. of such a Donnan system were determined from total volume

less A.O.V., there would be changes of A.F.S./F.S. with concentration. The concentration of the salt entering into the free space would be less than that outside and still less the lower the concentration, and hence the A.O.V. would be greater. The rise in A.F.S. with increasing concentration shown in Table I may be partly of this nature. If the cation balancing A were *k* and the density of a solution of *k* Cl were less than that of a solution of KCl of equivalent concentration, then the A.O.V. would be less.

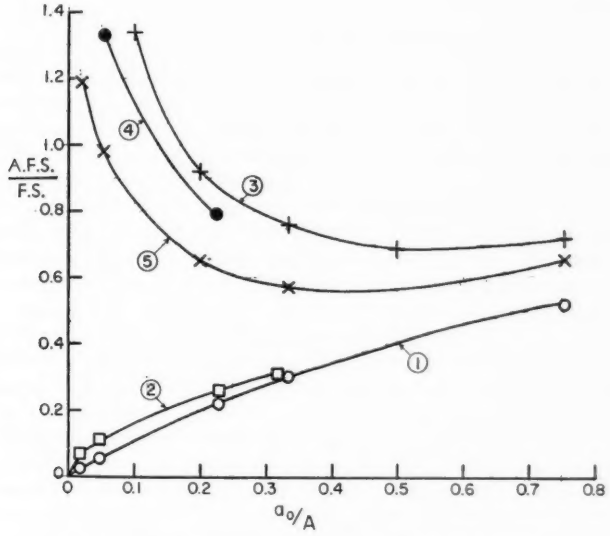

FIG. 2. Relation between the quantity A.F.S./F.S. and the concentration of salt in the external solution.

(*c*) Entry of weak electrolytes. If the solute in the external solution is the salt of a weak acid (Ha), the pH of the external solution will have an effect on A.F.S. calculated from the total amount of a^- (whether as Ha or a^- ion) passing from the solution into the free space containing a nonmobile anion. The following numerical illustration shows how the ratio of A.F.S./F.S. falls as the external pH rises while the total amount of Ha and a^- outside is constant. If the external concentration of $Ha+a^-$ is half the concentration of A in the Donnan system, the ratio A.F.S./F.S. falls from 0.82 when one-fifth of the total acid is ionised to 0.62 when half is ionised and then to 0.48 when four-fifths is ionised. For twice the external concentration, the comparable values of the ratio are 0.84, 0.73 and 0.64.

It must also be remembered that as the external pH is increased, the in-

XUM

ternal pH which is less than the external, will increase to a greater extent and there will be a consequent increase in the concentration of A^- by ionisation of HA. The extent of this will depend upon the acidic dissociation constant of HA. This increasing ionisation of HA will tend to increase the fall of A.F.S./F.S. as the external pH is increased. If when four-fifths of the acid in the external solution is ionised, the amount of A^- is doubled, the ratio of A.F.S./F.S., which is given above as 0.64, is reduced to 0.48. In our other illustrations from Donnan systems we have not included changes of ionisation of HA.

Properties of Free Space

Concentration of A.—Determination of the concentration of A is clearly a matter of some importance but is difficult to achieve. Some of the methods which may be used and their inherent limitations must be discussed.

1. Determinations from potential differences (P.D). If a system containing a nonmobile anion of concentration A is in contact with a solution of a salt such as KCl of concentration C_1 at one point and a concentration of nC_1 at another point, then from the potential difference between reversible electrodes immersed in the two solutions of KCl as a function of C_1, it is possible, given certain assumptions, to calculate both A and the ratio, μ_k/μ_a, of the mobilities of K and Cl.

Several such estimates have been made [Meyer & Sievers (19); Meyer & Bernfeld (18); Tendeloo, Vervelde & Zwart Voorspuij (22); Hope (11)].[2] The assumptions implicit in the estimates of Meyer *et al.* are that A is uniform throughout the system and that the only mobile ions are the same as those supplied in the external solution. As pointed out by Tendeloo *et al.* if the pH of the two external solutions is the same, the concentration of H ions in the system at the place of contact with the stronger solution will be less and the concentration of A greater than for the weaker. Assuming that $\mu_K = \mu_{Cl}$, Tendeloo *et al.* estimated both A and the acidic dissociation constant of the acid HA.

A feature neglected by all the above workers is the possibility of the presence of other mobile cations. These will affect the potential difference and hence the calculated value of A if their valency is different from that of the cation supplied in the external solution.

There is good evidence that the cytoplasm of plant cells contains mobile bivalent cations and, since in a Donnan system the ratio of bivalent cations inside to out is the square of that for univalent cations, it is much more difficult to remove the bivalent than to remove univalent cations by washing

[2] Lundegårdh in his early papers (15) interpreted his results in terms of ions being fixed on the surface of the cytoplasm rather than in the cytoplasm. Simple calculations show that there is not enough room on the surface of the cell for all the mobile ions which pass into the free space. More recently Lundegårdh (16) has accepted the idea that the ions do pass into the free space in the cytoplasm.

in water or by immersing in a solution of a uni-univalent salt. Neglect of bivalent cations reduces the estimate of A as the following examples show.

If a Donnan system with A balanced by a bivalent cation is placed in a solution of uni-univalent salt (say KCl) of volume 240 times that of the Donnan system and with a concentration of 0.004 that of A, when equilibrium is reached the value of Cl_0/Cl_i is 40. For a concentration of KCl very high relative to A, the ratio approaches unity. Hence if two such Donnan systems were connected and the diffusion potentials neglected, the total P.D. would be 93 mv. If there were only univalent cations present, a P.D. of this value would be given with the dilute solution 0.025A and hence the assumption that only univalent cations were present would result in a calculated value of A only 16 per cent of the true value. If the volume of the external solution were reduced to a quarter, the calculated value of A would be only 6.5 per cent. This calculation assumes that the volume of the Donnan system remains constant. If it was enclosed in a plant cell so that its osmotic pressure remained constant (cf. p. 23) then as the bivalent cations were replaced by univalent the osmotic pressure would tend to rise and hence the volume of the Donnan system would tend to increase and the consequence would be that the P.D. and the estimate of A would be greater.

Hence in any attempt to estimate A from P.D. it is advisable to arrange for the system not to have the above complication of replacement of the mobile cations by cations of different valency.

There are so many unknowns in the experiments on P.D. from which estimates of A have been made that it is impossible to conclude by how much A may be underestimated. It should be realised, for instance, that when in a biological system the connection between measuring electrodes is not wholly via a single phase such as the cytoplasm (but also contains water filled spaces and walls) then it is impossible to estimate the concentration of A in the cytoplasm.

2. Determination from the ratio of inside to outside concentrations of an ion. For a simple Donnan system with only one mobile cation and one mobile anion, the value of A can be calculated when the concentrations of the mobile anions inside, C_I, and of the anion and cation outside are known.

Since

$$C_i(C_i + A) = C_0^2$$

$$A = \frac{C_0^2 - C_i^2}{C_i}$$

but since it is the amount of anion, a, which has entered the free space and not its concentration which is known, the equation becomes

$$\frac{a}{v}\left(A + \frac{a}{v}\right) = C_0^2$$

where v is the volume of the free space.

XUM

Hope (11), using values of a obtained for different values of C_0, determined A and v. These estimates are valid if (a) there is only one kind of mobile cation in the Donnan system, (b) v does not change with C_0, and (c) all the free space is in the Donnan system. Hope considered only the last point. His conclusion that free space extra to that in the Donnan system is negligible depends upon whether his implicit neglect of the two former points is justified. Neglecting (b) and (c), the estimate of A refers really to $A-X_i$ where X_i is the internal concentration of mobile cations other than those supplied in the external solution. This may be relatively large if they are bivalent and will become yet larger as the external solution is made more dilute and the volume of the external solution is made smaller. For example, if a Donnan system originally had A balanced wholly by a bivalent cation and was placed in a solution of concentration 1.2 per cent of A and of volume one hundred times its own, then $A-X_i$ is only 24 per cent of A. We shall return later to the possibility of the variation of v with C_0.

3. Determination from data on exchangeability of cations and anions.

Briggs (4) has given equations from which the value of A can be calculated from the amount of cation exchange between a Donnan system if the ratio (v_e/v_0) of the volume of the system to the outside and the valency of the mobile cations in the system are known.

The results obtained by Sutcliffe (21) with slices of beet in solutions of KBr at 5°C., where accumulation is so slow that a good estimate can be made of the K and Br entering the free space, indicate an exchange of 11.6 per cent of the K when the external concentration of Br is 19×10^{-3} equiv./l. We may assume that the mobile cation originally inside was bivalent since the tissue after prolonged washing in distilled water had no readily exchangeable K. From this a value of A of 0.83 equiv./l. is obtained if the cytoplasm occupies 4.2 per cent of the tissue and 0.27 if it occupies 8.4 per cent. Since the amount of Br entering such a system is a very small fraction of that which enters the whole free space, 4.5×10^{-3} equiv./kg., then if the rest of the free space is water with no nonmobile ions it occupies about 4.5/19 or 24 per cent of the tissue. This is, by definition, also the value of A.F.S.

The results of experiments by Stiles & Skelding (20) with carrot tissue are more useful since they used a range of concentrations of external solution which enable several estimates of A for comparable samples of the same tissue.

Assuming that cation taken up in excess of anion into the free space is exchanged with cation in a Donnan system, then if the cation originally in the system and that in the external solution have the same valency as the anion in the latter, the total equivalent amount of nonmobile anion, Av, can be calculated no matter what the value of v relative to that of the external solution. If the cation originally in the carrot cytoplasm was bivalent, then from Mn exchanged by 200 ml. of 20×10^{-3} M $MnSO_4$ with 18.9 cc. of carrot, we calculate 32×10^{-3} equivalents of A in 1000 cc. of tissue. For the experiment with 5×10^{-3} M, the value for total A is 23.2×10^{-3} equivalents and for 1×10^{-3} the value is 19.6×10^{-3} equivalents per 1000 cc. The amount

of SO_4 ions absorbed is too small to estimate the value of v. If this is only 5 per cent of the tissue then the corresponding concentrations of A are 640, 464 and 392×10^{-3} equiv./litre. If some of the mobile cation in the Donnan system was univalent, then the estimates for A are too high and increasingly so for the lower concentrations of $MnSO_4$. Even if the fraction which is univalent is known, the concentrations of A cannot be calculated unless the volume, v, of the Donnan system relative to that of the external solution is known.

Higinbotham & Hanson (10) state that the relation between the logarithm of the amount of Rb entering the free space of potato discs (6 gm. of tissue in 100 ml. solution) and the logarithm of the concentration of RbCl is linear and make reference to the Freundlich isotherm. In fact, their statement holds approximately only over a tenfold range of external concentrations. The same is true for a Donnan system placed in a solution of uni-univalent electrolyte over a considerable range of ratio of volume of solution to volume of Donnan system and holds whether A is balanced by univalent or bivalent cation.

Volume of free space; change of free space with external concentration.—If the cytoplasm of the plant cell behaves as a Donnan system, then, since the sum of the concentrations of mobile ions inside must exceed the sum of the concentrations outside, the cytoplasm will tend to swell. If the hydrostatic pressure on the cytoplasm is the same as that on the vacuole, in other words, if the cytoplasm behaves as a fluid, then, if the cell wall has negligible extensibility, the cytoplasm will have a minimal volume when the cell is in water and when the water is replaced by a solution with a solute which penetrates the cytoplasm, but not the vacuole, the cytoplasm will increase in volume as much as the vacuole decreases. If the cytoplasm has a structure which tends to resist expansion, then, although the volume of the cytoplasm will increase at first, it will not continue to occupy the whole of the space between vacuole and cell wall; in other words, plasmolysis will take place. Should the solute weaken the structure of the cytoplasm, then it will swell again and fill the space up to the wall. This Kappen-plasmolysis occurs with many cells in solutions of salts such as KCNS, and with some in KNO_3.

The following numerical examples will illustrate the possibilities. A cell with a relatively inextensible wall has a cytoplasm with a volume negligible compared with that of the vacuole. If the amount of univalent cation is 10 and the volume of the cytoplasm unity when the cell is in water, then, when the cell is placed in a solution of a uni-univalent salt, of concentration 4, that is with an osmotic pressure about 80 per cent of that of the vacuole, the following equations must be satisfied:

$$\frac{10 + a}{v_c} \cdot \frac{a}{v_c} = 4^2$$

$$\frac{10 + a}{v_c} + \frac{a}{v_c} = 10$$

v_c is the volume of the cytoplasm and a is the amount of anion, and the

amount of cation, entering. The first is the Donnan equation and the second is that expressing equality of osmotic pressure of cytoplasm and vacuole—the volume of the latter being so large relatively that change in it may be neglected. The new volume of the cytoplasm v_e is 1.68, i.e., it has increased 68 per cent. If the volume of the cytoplasm when the cell is in water was one ninth of the volume of the vacuole the increase would be about 50 per cent. If A.F.S. had been calculated from the amount of anion entering, *a*, then the A.F.S. would have been about 85 per cent of the original volume of the cytoplasm, i.e., only about half the new volume. If the cytoplasm had retained its original volume, A.F.S./F.S. would have been about 35 per cent.

If the cation originally in the cytoplasm were bivalent then in consequence of the replacement of these by univalent cations from outside the osmotic pressure of the cytoplasm would tend to increase further and there would be more swelling. The external volume being very big, practically all the bivalent cation would be replaced by univalent and the cytoplasm would extend indefinitely unless restricted by the increasing O.P. of the vacuole. If the original volume of the cytoplasm was one-ninth of that of the vacuole the new volume of the cytoplasm would be about 4.6 times its original volume, and the A.F.S. three times the original volume.

There is a further complexity for which we have made no allowance. The cell wall is not rigid and the volume of vacuole plus cytoplasm will be reduced when placed in the solution by an amount dependent upon the extensibility of the wall and the structure of the tissue.

If, however, when the cytoplasm (behaving as a Donnan system) expands, an inward pressure is exerted on the solution in the free space of the cytoplasm as a result of a strain in the cytoplasm, then after the inward pressure via the cell wall has been reduced to zero, further increase in the external concentration will result in a decrease in the volume of the cytoplasm. The inward pressure may be assumed for the sake of argument to be proportional to the volume (v_e) or to $v_e - X$, where X is a constant. In the former instance (where $X=0$) the A.F.S. deduced from the entering anion would first rise with external concentration and then fall as the volume of v_e fell. The ratio A.F.S./F.S. rises throughout. If X is finite, then A.F.S. would continue to rise up to the limit X. In arriving at the above conclusion we have assumed that the strain in the cytoplasm has resulted in negligible pressure or tension on the vacuole. Again, the volume of the cytoplasm and the A.F.S. would depend upon whether the cation in the cytoplasm originally had the same valency as that outside or different.

When considering estimates of A.F.S. from the results of experiments using sugar solutions, the probability should be borne in mind that the free space in the cytoplasm is decreased by sugar as is the free space in a gelatine gel by cane sugar.

Butler's (7) estimate of A.F.S. in wheat roots using mannitol is 24 to 25 per cent of the tissue. His estimate using labelled phosphate is about 33 per cent. Since he uses total uptake in half an hour and does not allow for slower

uptake which continues, the latter estimate is too big by about a tenth. As we have seen the A.F.S. of a Donnan system determined by the anion entry from an electrolyte should be smaller than that from the entry of a nonelectrolyte if the volume of the free space is not affected. Hence we must conclude either (*a*) that the volume of the free space in the Donnan phase is reduced by mannitol, or (*b*) that the bulk of the free space is extra-cytoplasmic (non-Donnan), or (*c*) that the rapid uptake includes phosphate which has been bound or a combination of (*a*) and (*c*). Some indication in favour of (*c*) would be if its transfer at an early stage to a large volume of unlabelled phosphate failed to remove the labelled phosphate from the tissue.

Epstein (8) has estimated the A.F.S. (he calls it "outer space") in excised barley roots to be about 25 per cent of that of the tissue. Since the basis of the estimate was the amount of anion or cation eluted by water, the A.F.S. is a measure of the space occupied by water with no nonmobile anions plus a fraction of the Donnan space—a fraction equal to the ratio of the mobile anion concentration in the Donnan system to that in the external solution, a fraction which is likely to be small for the conditions of his experiments and very small for bivalent anions.

The term "outer space" as used by Epstein apparently refers to that part of the free space where cations and anions enter equally. It is measured by the uptake of anions when their external concentration is small compared with A, with the consequence that little anion penetrates the Donnan part of the free space. It would have a different value if measured with nonelectrolytes which could penetrate the Donnan part. He does not allow for this part of the free space. His outer space is "accessible by diffusion," his "inner space" is "the region to which ions are transported by active carrier mechanisms." The Donnan part of our "free space" is also accessible by diffusion.

Osmotic pressure of the cytoplasmic component of the free space.—In our previous discussion, we have assumed that the vacuole is exposed only to the inward pressure transmitted via the wall, partly due to the stretched wall and partly due to the surrounding tissue. If the same applies to the cytoplasm, then the osmotic pressure of the vacuole and cytoplasm must be equal. If the contents of the free space in the cytoplasm are under an extra pressure due to strain in the cytoplasm then the osmotic pressure of the cytoplasmic phase must be greater by the amount of the extra pressure. We are assuming that there are no metabolic processes tending to transfer water between cytoplasm and vacuoles: evidence for such processes is in our view open to other interpretations [Mercer (17)].

On the above basis it is possible to arrive at an estimate of A and v_e from data such as those of Sutcliffe (cf. p. 22). From the exchange of K we calculated, on the assumption that the cation originally in the cytoplasm was bivalent, that $A = 0.27$[3] if v_e was 8.32 per cent of the tissue. From this and the concentration of K it can be deduced that the concentration of univalent

[3] Concentrations of A are in equiv./liter.

cation in the cytoplasm would be 0.13 equivalents per litre of cytoplasm and of the bivalent 0.14; that of the Br is negligible. This gives an O.P. for the cytoplasm, if there are no other osmotic substances,[4] equal to that of 0.1 *N* KBr. Similar calculations give values of O.P. equal to that of 0.27 *N* and 0.75 *N* for v_e equal to 4.16 per cent and 2.08 per cent respectively. If the O.P. of the vacuolar sap is known to be equal to that of a concentration of KBr of say 0.2 *N*, then from curves of calculated O.P. and A against v_e the value of A and v_e equivalent to an O.P. for 0.2 *N* can be found. The figures in this case are about 0.55 for A and 5 per cent for v_e, where the cytoplasm is 1 μ thick, if its radius is 60 μ. Extra hydrostatic pressure on the cytoplasm would increase A and lower v_e; presence of other osmotic substances in the cytoplasm would have the reverse effect.

Hence the assumption that the osmotic pressures of cytoplasm and vacuole are equal and the accompanying assumption that the osmotic pressure of the cytoplasm is due mainly to mobile cations, which might seem surprising, are not to be lightly ruled out as unreasonable.

Equilibration of the free space with an external solution.—The time relations of the penetration of solutes into the free space depend not only upon the mobility of the solute in the free space but also upon the geometry of the free space. If the solute is an electrolyte there is the question of gradients of electric potential as well as gradients of concentration except when the mobilities of the different ions are all the same.

Briggs (4) has considered the following cases. For a Donnan system of volume v and area x where the diffusion inside the system can be neglected the rate of change of concentration C is

$$\frac{dC}{dt} = \frac{D \cdot x}{d \cdot v}[C_0 - \sqrt{C(C + A)}]$$

The effective length of the path, d, depends on the geometry of the system and the stirring of the outside solution.

If the diffusion gradients are limited to the free space and this is uniform in shape and composition in planes to which the direction of diffusion is normal then, if at zero time the space is full of water or a Donnan system containing A balanced by K, the uptake of KCl, X, assuming the mobility of K and Cl equal, will follow a time course which results in the slope of the curve $\ln (X_\infty - X)$ against time approaching very closely to constant value by the time $X = 0.5X_\infty$. If the tissue is in the form of a relatively large thin disk of thickness l this slope is $8D/l^2$, if in the form of a relatively long narrow cylinder of radius r the slope is $5.9D/r^2$ where D is the coefficient of diffusion in the substance filling the free space. The half-times are $l^2/20D$ and $r^2/16D$

[4] Admittedly there must be some molecules of metabolic substances in the cytoplasm, but until there is evidence to the contrary, we do not believe there is an appreciable concentration of these in the free space if they do not appear in the external solution.

for disk and cylinder respectively. For $l = 0.1$ cm. and $D = 1 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ the half-time is 50 sec. If the free space is of variable cross section, then the slope should be less (the shape of the free space is too complex to say by how much) and might never reach a steady value. If the free space is occupied partly by water and partly by a Donnan system the equilibration is more complex. If the concentration of KCl is small relative to A then little KCl will enter the Donnan system. The equilibration follows the same course if the free space is in equilibrium with a salt such as KBr and a small trace of labelled ion is added to the external solution. The results of the experiments of Hope (11) with bean roots in KCl suggest a value of D from the steady slope one-quarter of the value in water and from the half time a value of the order of that for water. This may be because the bulk of the free space is near the periphery.

If in the free space consisting of a Donnan system, or water, there is a special phase in which the KCl is accumulated, the equilibration follows the same course if this special phase equilibrates relatively rapidly with the rest of the Donnan system, except that the constant is no longer D, the coefficient of diffusion, but D/σ where σ is the ratio of the average concentration in the whole Donnan phase plus special phase to that in the Donnan phase alone. If σ is a function of concentration then the equilibration is more complex. If, on the other hand, the equilibration of the special phase is very slow relative to the diffusive equilibration of the rest of the free space the former process will set the tempo of equilibration of the whole. If the tissue is in equilibrium with a salt solution and one of the ions is labelled and ρ is the turnover rate between the Donnan phase (or water) and the special phase then, if the specific activity of an ion outside is made S_0, the specific activity of the ion in the whole free space, S_t, will be given by

$$S_t = S_0(1 - Re^{-\rho t/k_f})$$

where k_f is the amount of the ion in the special phase, R is the ratio of this to the total amount in special phase plus the remainder of the free space, t is time, and e the base of natural logarithms.

Krebs, Eggleston & Terner (14) applied this equation to determine ρ for potassium in slices of brain tissue having assumed that R is unity and the whole tissue is free space. They did not make the obvious investigation of the effect of thickness of slice on the rate of equilibration to test their assumption that diffusion could be neglected. If we assume that the tempo of equilibration is set by diffusion and that the tissue is all free space the asymptotic value of the slope of the curve of $\ln (S_0 - S_t)$ against t is $8D/\sigma l^2$ instead of ρ/k_f, as they assume. This gives a value of D equal to about one-eighth of the value in water. If only half the tissue were free space then σ and D would be nearly twice as great, that is, not very different from the value calculated for bean roots on the assumption that diffusion is the pace-making process in the equilibration of this tissue.

Relation to morphological constituents of cells and tissues.—There is much evidence that the free space of the tissue for electrolytes and molecules such as sucrose comprises the injected intercellular spaces, wet cell walls, and much of the cytoplasm. There is also much evidence from experiments on isolated protoplasts, on isolated vacuoles contained within tonoplasts or tonoplasts with little adhering cytoplasm, on plasmolysis, and on slow leakage rates in the presence of substances which accumulate, that the tonoplast has considerable resistance to the diffusion of electrolytes and of molecules like sucrose. The free space of the tissue can therefore probably be regarded as the intercellular space, wet cell wall and, for many solutes, the cytoplasm surrounding the tonoplast. The division between cell wall and cytoplasm is probably not clear-cut either physiologically or morphologically. This cytoplasm may be regarded as a gel which has some structural rigidity due to the micelles of cytoplasmic protein and containing nonmobile anions as part of this structural framework. In addition, allowance must be made for the fact that the cytoplasm may be multiphasic, i.e., that there are lipoid regions or aqueous regions surrounded by lipoid (as in mitochondria) which are separated from the aqueous phase of the gel and which may fix solutes metabolically or otherwise. If such phases adjust readily in salt concentration with that of the aqueous phase of the gel, they will be included in the estimate of apparent free space. If however these phases adjust only slowly, they will be included in the estimate of apparent osmotic volume. Nothing is known of the times of adjustment of mitochondria in intact tissue, but mitochondria extracted from red beet tissue adjust themselves to changes in salt concentration with half times of about 2 min. If the times of adjustment within the tissue were similar, mitochondria would be included in the free space. Substances affecting fixation by mitochondria would affect A.F.S.

Significance of free space in plant cell physiology.—If the picture we have drawn of the cytoplasm in relation to the external solution is correct, then the measure of the activity of substances which are being consumed or produced in metabolism or being transported between external solution and vacuole is not the average concentration in the tissue. For metabolites, it is the concentration at the seat of the reaction in the cytoplasm which determines the metabolic rate. If the reactants (e.g., sugars) come from the vacuole, the rate at which they pass through the tonoplast is an important factor; if the products are electrolytes (organic acids) the rate at which they are accumulated into the vacuole will be of importance in determining the ratio of vacuolar concentration to the effective concentration in the cytoplasm. The accumulation of solutes supplied externally being slow compared with their entry into the cytoplasm, their activity in the external solution will be a better measure of their activity in the cytoplasm than is the average concentration in the tissue as a whole. This is relevant both for the rate of accumulation and for the rate of metabolism. A much higher rate of respiration may be obtained with a given concentration of metabolite in the external

solution than with the same average-concentration in the vacuole [cf. Bennet-Clark & Bexon (3); Turner & Hanly (23)]. The relation between the rate of accumulation and the activity of a solute in the external solution (which is the same as that in the free space) will depend upon the mechanism of accumulation of which we know little.

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MINERAL NUTRITION OF PLANTS[1,2,3]

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INTRODUCTION

Because of the great number of publications on mineral nutrition, the following review deals primarily with the higher, green plants. It covers research in mineral nutrition for the last two years inasmuch as the excellent, current review by Epstein (55) was limited to salt absorption. Special attention is called to other reviews dealing with the use of tracers in mineral nutrition [Biddulph (12)], iron chlorosis [Brown (28)], phosphorus metabolism and photosynthesis [Arnon (9)], and nitrogen fixation by photosynthetic organisms [Fogg (65)]. The function of metallo-flavoproteins in inorganic nitrogen metabolism was discussed recently in a symposium and subsequently published [McElroy & Glass, Eds. (125)]. Lal & Rao (106) have reviewed the micro-element nutrition of plants with an impressive coverage of literature.

SALT ABSORPTION PROCESS

Absorption by Roots

Aeration.—Confirming earlier reports in the literature, Hammond *et al.* (79) found that either low O_2 tension or high CO_2 in the root environment drastically reduced the absorption of potassium. Oxygen and CO_2 combinations, such as they found were associated with reduced K absorption, did not occur under field conditions—at least, as indicated by gross analyses of soil atmospheres. They postulated, however, that the micro-climate of roots

[1] The survey of the literature pertaining to this review was concluded in September, 1956.

[2] Abbreviations are as follows: adenosine mono-, di-, and triphosphates (AMP, ADP, and ATP, respectively); ethylenediaminetetraacetic acid (EDTA); indoleacetic acid (IAA); indoleacetic acid oxidase (IAA oxidase); adenylic kinase (AK); reduced and oxidized di- and triphosphopyridine nucleotides (DPNH, DPN, TPNH, and TPN); flavin-adenine dinucleotide (FAD); flavin mononucleotide (FMN); and, 2,4-dichlorophenoxyacetic acid (2,4-D).

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XUM

might involve O_2 and CO_2 concentrations that would curtail K uptake by plants. Hopkins (91) reported that P absorption was independent of O_2 tension over the range from 3 to 100 per cent, where the total pressure was one atmosphere. Over the range from 3 to 0 per cent O_2 tension, the relation was typically hyperbolic, and at 0.3 per cent the absorption rate of phosphate was half-maximal. The first order reactions for both H_2PO_4 and HPO_4 had O_2 requirements. It is possible that the above effects of O_2 or CO_2, or both on K and P absorption are related to the fact that solute entry depends on "carriers" which are formed and maintained by virtue of the metabolism of root cells. Although the author is unaware of similar recent studies with other ions, it would appear likely that the active absorption of any ion would be reduced by low O_2 or high CO_2 concentrations around the roots. Earlier studies have indicated that ions accumulated in excess by roots may be lost to the external solution under conditions of O_2 insufficiency.

"Carrier concept" and exchange adsorption.—According to Epstein (54, 55, 57) and Hylmö (92), ions may enter roots by diffusion, through "exchange adsorption," and also by the action of carriers, or ion-binding compounds. According to Epstein's (54) carrier concept, "ions combine with carrier molecules and the resulting ion-carrier complexes traverse membranes of limited permeability to the free ions. At the inner surface of the membranes, the ions are released from the carriers." In addition to this mode of entry, "cations may be nonmetabolically adsorbed on negatively charged surfaces within the root, in stoichiometric exchange for other cations residing on these exchange surfaces" (54).

The ions which are nonmetabolically absorbed by diffusion and exchange adsorption are believed to exist in the "outer space" of the cells. Epstein (54) characterized the "outer space" and the state of the ions in it, as follows:

> The apparent free space represents about 23 per cent of the volume of barley root tissue. At equilibrium, the concentration of the ions in the "outer" space of the tissue equals their concentration in the ambient medium. There is no competition among different ions for the "space." There is no pH effect. The "space" was freely accessible to selenate, phosphate, and calcium ions, in addition to sulfate.

Combining Epstein & Leggett's (53) characterization of these two mechanisms for ion entry with additional features listed above, one might summarize these two modes of entry as follows:

Exchange Adsorption	Active Absorption
Diffusion and adsorption process	Ion-binding compounds, or carriers, are involved
Nonlinear with time; equilibrium approached within an hour	Linear with time; no equilibrium reached in the experiments
Ions readily exchangeable stoichiometrically for other ions	Ions essentially nonexchangeable

Not highly selective with respect to various ions; hence fairly indiscriminate competition among ions with respect to entry	Selective with respect to various ions and groups of ions; hence highly specific with regard to competition for site and entry
Does not require expenditure of energy on part of root; nonmetabolic	Requires expenditure of energy; depends on metabolism (aerobic)
Ions are in adsorbed state in "outer space" of cells	Ions presumably in vacuoles

With these two modes of ion uptake fully outlined, it is pertinent to attempt to reconcile with them certain recent observations. Tanada (174) reported that Ca^{++} ions greatly enhanced the uptake of both Rb and P, and that there was no adequate explanation for the stimulatory effect of calcium. Leggett & Epstein (109) also noted a stimulatory effect of Ca on SO_4 absorption by excised barley roots. The most favorable level of Ca in the solution caused a maximal activation (of SO_4) of 203 per cent of the controls. Leggett (110) has recently obtained evidence which indicates that Ca acts by virtue of increasing the "turn-over" rate of the site involved with HPO_4 absorption.

In connection with K absorption and assuming that K absorption involves the reaction, $K^+ + HR \rightleftharpoons KR + H^+$, Nielsen & Overstreet (144) deduced that increased concentrations of K^+ or HR (R = the protoplasmic constituent) should increase absorption of K^+, whereas increased concentrations of KR or H^+ should decrease it. Experimentally an increase of H^+ ion was found to decrease K absorption. It was further reasoned that if absorption of an ion is a function of the ionic concentration at the site of the cation-binding reaction, then the addition of Ca should reduce the absorption of potassium. Actually the addition of Ca increased K absorption. They concluded that, under the conditions of their experiments, Ca was acting as a co-factor in the utilization of the K complex, KR. Such an action of Ca in removing the complex, KR, would tend to drive the above reaction to the right and hence to stimulate K absorption. Although no direct evidence for this role of Ca was presented, this type of action would be consistent with the often reported stimulatory effect of Ca and other polyvalent cations on ion absorption.

With regard to the action of carriers, Ordin & Jacobson (149) have proposed that ATP may be involved in ion absorption by reacting with intermediates of the Krebs cycle to form ion carriers or to destroy them. According to this view, metabolism would be required both to provide ATP and to provide intermediates which may interact with ATP to function as ion carriers. Higinbotham & Hanson (84) favor the carrier concept, but they are of the opinion that in initial uptake, R (the protoplasmic constituent) may not represent a single substance behaving stoichiometrically.

Tanada (174, 175) has postulated that the carriers are ribonucleoproteins with the nucleic acid binding the cations and the protein moiety binding the

XUM

anions. Steward & Street (169), on the other hand, postulated that the carriers are phosphorylated energy-rich nitrogen compounds which would presumably be formed by aerobic respiration. They attempted to rationalize this postulate, concerning the carriers, with respiration and protein synthesis. They suggested that the carrier is broken down in the process of its incorporation into protein (at the seat of protein synthesis) and that simultaneously the associated ion or ions (which it was carrying—cation or anion, or both) would be released in free solution into the vacuole. According to their theory, ions are released by protein synthesis and energy-rich nitrogen compounds, as carriers, are presumably formed by aerobic respiration.

In connection with exchange adsorption, Smith & Wallace (166) were of the opinion that widely differing cation-exchange capacities of two species could have a pronounced effect on the natural competition between the two species. They cited work which indicated that the compatibility of grasses with legumes was positively correlated with the cation-exchange capacities of the roots of the grasses. They cited other research which indicated that the cation-exchange capacity of dicots is nearly twice that of monocots. In view of this observation, it is difficult to understand the outstanding capacity of barley and many other grasses to grow on soils of low fertility.

McLean *et al.* (128) observed that there was a relationship between the tendency of a plant to absorb Na and the bonding energy of Na to the plant root. Legumes were found to have high bonding energy values for Ca; grasses, high values for K—so high, in fact, that they would take K even to the detriment of the legumes. They also noted that there is apparently a relationship between the ability of a plant to utilize P from rock phosphate and the relatively high mean free-bonding energy of the roots for calcium.

Elgabaly (48) concluded that the uptake or depletion of a given ion by plants is modified by the nature of the complementary ion. Calcium depletion occurred at 82 per cent Ca-saturation when Na was the complementary ion and at 28 per cent when Mg was the complementary ion. He concluded that these differences could be explained on the basis of the Donnan membrane theory and by differences in the activities of adsorbed ions.

Jacobson (95) reported that there are profound changes in organic acids concomitant with cation and anion absorption. Of the $^{14}CO_2$ which excised barley roots fixed under conditions of their experiment, most of it appeared in malic acid—in the case of excess cation absorption, 92 per cent of the accumulated C^{14}.

Current status of carrier concept.—The concept of ion-binding compounds as carriers appears to be widely accepted (50, 55, 57, 67, 84, 89, 144, 149, 177, and others). Using Lineweaver and Burk plottings of data or other evidence, various interionic relationships in salt absorption have been determined. Potassium, Rb, and Cs compete for the same site, whereas Na and Li do not compete for the former site [Epstein & Hagen (49)]. With Na and K passing through membranes over different sites [Epstein & Hagen (49); Scott & Hayward (158)], selectivity by species with regard to these two ions

comes into focus. In view of possible differences in concentrations of the sites concerned in Na and K absorption, it is also possible to visualize conceptually why certain plants are "Na accumulators" and others are "K accumulators." Thus, among a group of ions that compete with each other for sites, a mechanistic picture is available concerning at least one type of "antagonism."

Similarly, Epstein & Leggett (53) have found that Ca, Ba, and Sr compete for identical sites; that Mg passes over a different site. Hagen & Hopkins (78) demonstrated that H_2PO_4 and HPO_4 enter over different sites, but that the OH^- ion interfered competitively with both sites and hence both forms of phosphate. Leggett & Epstein (109) showed that SO_4 and SeO_4 compete for the same site. Phosphate, NO_3, and Cl were found to have no measurable affinity for the SO_4-SeO_4 binding site. Calcium increased the rate of SO_4 absorption, but the nature of this activation was undetermined. Epstein (50) found that Cl competed with Br but that NO_3 did not compete for the Br site. Van den Honert & Hooymans (89) postulated that there is a separate carrier for NO_3, that NO_3 absorption is much greater at pH 6 than at 8, and that the HCO_3^- ion had no effect on NO_3 uptake. Active transport of all ions, therefore, apparently involves their combination with protoplasmic constituents, and this then forms the chemical basis for selectivity [Epstein (50, 51)]. Foulkes (67) recently reported evidence that in yeast there exists a "carrier" for K uptake but not one for "Na extrusion." The concentration of the K carrier was calculated to be 0.1 μM/gm. of yeast.

Chemical nature of the carriers.—Although the nature of carriers is still unknown, various compounds have been suggested. Steward & Street (169) postulated that phosphorylated energy-rich N compounds function as carriers. Lansing & Rosenthal (107) and Tanada (174, 175) favored the ribonucleoproteins as carriers, and Tanada (174, 175) suggested that the nucleic acid portion binds the cations while the protein moiety binds the anions. There seems to be complete agreement that ion-binding compounds must be capable of readily undergoing oxidation and reduction, or of undergoing some other change in energy level such as would be involved with phosphorylated compounds.

Bioelectric Potentials.—By means of an elecdropode, Breazeale & McGeorge (23, 24) subjected plants to half-wave voltage potentials ($E\frac{1}{2}$) of 2.10, 2.13, 2.20, and 2.23 which are more or less specific for Na, K, Ca, and Mg, respectively. The voltage which was appropriate for a given cation increased the uptake of the ion and, for the first few days of the experiments, stimulated the uptake of the element whose "most favorable voltage" was closest to that of the given voltage being applied. They concluded, therefore, that bioelectrical potentials play an important part in cation uptake by growing plants. It is not apparent how these effects can be reconciled with the well established concept of ion-binding compounds as carriers of ions in salt absorption.

"Salt respiration."—Many workers have observed that the addition of a

XUM

deficient element to plant tissues, roots in particular, causes an increase in respiration—so-called "salt respiration." Lewin (112) recently observed a similar effect upon the addition of silicate to Si-deficient *Navicula pelliculosa*.

Lundegardh (116) stated that respiration is far more intimately related to the absorption of anions than to the absorption of cations, and that he therefore favored the term "anion respiration" to that of "salt respiration." His theory was formulated around the assumption that the absorption of anions involved a direct relationship to respiration and that the cations were "passively" attracted into and through the cells by virtue of potentials created by the active absorption of anions. Recent research by Epstein (52), however, indicates that the absorption of cations may also cause an increase in respiration. When excised barley roots were exposed to K^+ ions on a cation exchanger, there was observed a "cation-induced respiration." The conclusion was that cation and anion respiration are not fundamentally different processes. Honda (88) also reported that KBr stimulated the respiration of barley roots. Handley & Overstreet (80) reported that K and Na stimulated "salt respiration," but that Ca and Mg did not. They concluded, therefore, that the absorption of certain ions stimulates respiration whereas the absorption of other ions does not. It is possible that the degree of adequacy of a given ion in roots might determine whether or not an enhanced respiration would occur. In that connection, Harley *et al.* (81) obtained a close corrleation between phosphate absorption by excised beech mycorrhizas and oxygen consumption only when sufficiently high phosphate concentrations (more than 0.3 mM) were presented.

Working with washed beet slices, Middleton (130) recently observed that glucose, 3×10^{-5} M K-dinitrophenol, or KCl stimulated respiration and that cyanide negated the stimulation. With a low concentration or a complete absence of transportable ions, three very different substances increased respiration, suggesting that there was no direct stimulation of a particular terminal oxidase. He (130) concluded, therefore, that

> "the stimulated respiration may be cyanide-sensitive because the cyanide-insensitive system is of limited activity; if the rate of respiration is above the maximum this system can carry, it will be partially cyanide-sensitive. Any increase of respiration would be carried by the sensitive system."

New light is shed on "salt respiration" with this interpretation by Middleton (130) and by Epstein's (52) report of "cation-induced respiration."

Miller & Evans (131) reported that certain univalent and divalent cation salts may stimulate particulate cytochrome oxidase from soybean roots. Apparently, therefore, the activity of certain enzyme systems may be increased or decreased by various ions.

Although respiration was unaffected by Ca, Florell (63) observed that Ca favored the formation of mitochondria in roots. In view of this effect and the possibility that mitochondrial action is intimately linked through respira-

tion with salt absorption, an explanation would appear to be at hand for the enhancement of NO_3 absorption [Burstrom (34)] in the presence of calcium. The effect of Ca on mitochondrial formation might also be similarly related to the Ca-enhancement of HPO_4 [Leggett (110)] and SO_4 [Leggett & Epstein (109)] absorption. Many authors, among them Davies (40) and Robertson *et al.* (154), have concluded that many, if not all, of the Krebs cycle enzymes are associated with mitochondria. The presence of cytochrome oxidase in the particles is particularly well established. They state that the mitochondria hold both cations and anions in concentrations greater than those in the nonparticulate fraction (154). They further add that the mitochondria are probably involved in electrolyte accumulation.

Movement of Ions from Roots to the Tops

Contradictory evidence has appeared concerning the significance of the presence of the tops with regard to salt absorption by the roots. Hopkins *et al.* (90) stated that the transfer of ions from the roots to the shoots was independent of aerobic mechanisms in the root. Hylmö (92) and Wright & Barton (203) recently reported that the movement of ions into roots and, in turn, into the shoots is dependent on the rate of absorption of water (92) and of transpiration (203). Brouwer (25) reported that the correlation between water and ion absorption proved to be dependent on the salt status of the material. Salt uptake was found to be promoted by an increase in transpiration only when the absorbed salt could be utilized. Otherwise the rate of uptake was limited by the rate of utilization. Recent experimentation by Epstein (56) indicates that the ions which are actively absorbed enter essentially irreversibly into the vacuoles of root cells and that these ions are not therefore free to move to the tops. The evidence would indicate that, for the most part, the ions which move to the tops are those which are passively absorbed and exist in the "outer space" of the root cells. Although selenate interferred with SO_4 absorption by barley roots in a manner that could be predicted from experiments with excised roots, SO_4 transfer to the shoots did not appear to be affected in the same way by selenate [Epstein (56)].

Epstein (57) also reported that when excised barley roots were exposed to $^{35}SO_4$ and then suspended in humid air for varying periods of time before placing them in water, the amount of nondiffusable $^{35}SO_4$ steadily increased with time. That is, with time, more and more of the SO_4 that was in the "outer space" of the root cells was being "actively" and more or less irreversibly transported into the "inner space"—vacuoles and mitochondria.

TRANSLOCATION

General

With bean or cucumber plants in the dark from one to three days, absorption of ^{60}Co by the leaves occurred, but there was no translocation from the leaves [Gustafson (74)]. The addition of sucrose, glucose, fructose, or maltose to the isotope solution considerably increased the translocation of

XUM

^{60}Co. In the light, the translocation of Co was enhanced by an increase in light intensity or temperature and by the addition of S/V Sovaspray 100 to the isotope solution. Many examples might be cited of evidence to the effect that various essential elements may affect the movement of carbohydrates in plants, but Gustafson's (74) research indicates that sugars may, in turn, affect the translocation of an inorganic constituent. This is further indicated in a recent report by Gustafson & Schlessinger (75) to the effect that ^{60}Co entered but did not move from leaves of plants in the dark for 48 hr. Nightingale's (146) statement that the proportion of certain inorganic to organic components is just as important as proportionalities within inorganic constituents in plants is pertinent. Inasmuch as this downward flow of ^{60}Co presumably occurred in the living cells of the phloem, the sugar-dependent metabolism of the phloem cells would appear to be necessary for downward ion transport within the phloem. Biddulph (13) reported that downward movement of ^{35}S and ^{32}P in the red kidney bean plant occurred through the phloem.

With regard to translocation of ions from roots, Wiebe & Kramer (200) found that the region of greatest translocation of ions (^{32}P, ^{35}S, ^{45}Ca, ^{86}Rb, ^{131}I, and ^{90}Sr) was several centimeters behind the root tip—the region of maximum water absorption. Translocation from the region more than 50 mm. above the tip was somewhat less, but it was much greater than from the apical region of the root. That this region of the root should be the one most involved in the translocation of ions is compatible with the evidence presented in the section on "salt absorption" in which it was reported that there appears to be a relationship between the movement or flow of absorbed water and the movement of ions to the shoots. It is pertinent to Wiebe & Kramer's (200) work that Honda (88) observed a higher rate of "salt respiration" in the vacuolated region of barley roots, and that Brown & Cartwright (30) proposed that salt accumulation is a function of vacuolization and, particularly, of protein formation.

Specific Elements

Calcium.—Ferrell & Johnson (62) found that there was a substantial movement of previously deposited ^{45}Ca into newly developed buds in western white pine trees. Considering the specific activity of ^{45}Ca in foliage and buds at various periods after injection of ^{45}Ca into the trunk, the one year-old buds were found to have appreciably more ^{45}Ca than the foliage of the trees had shortly after injection. With the exception of this report of Ca mobility in a tree species, the other recent literature has dealt with herbaceous plants. For the latter, the recent reports reflect the generally held opinion that little or no movement of previously absorbed Ca occurs within the plant [Bukovac *et al.* (33); Bledsoe (16); Williams (201); Martin (120); Norton (147)]. Tukey (182) noted that foliar applied Ca did not move out of the leaves, and Wiebe & Kramer (200) reported that upward translocation of ^{45}Ca from the roots of barley seedlings was very limited. Similarly, Bledsoe

et al. (15) observed that, in the peanut plant, ^{45}Ca did not move from the fruiting medium to the leaves or stems, and that ^{45}Ca supplied to the rooting medium, on the other hand, did not move to the pegs. For the growth of the pegs, many studies, among them Bolhuis & Stubbs (17), have indicated that Ca must be available in the fruiting zone per se.

Bukovac *et al.* (32) reported that there was little or no transport of ^{45}Ca through graft unions of herbaceous plants involving a reversed polarity. When they negated the polarity of bean leaves by anesthetizing them with diethyl ether, considerable quantities of ^{45}Ca moved from the site of application to other parts of the plant. Inasmuch as Ca did not move against polarity but moved readily when polarity was negated, it would appear that Ca should normally move freely in one direction in a plant. It is difficult to reconcile this interesting report of considerable Ca mobility in a polarity-free plant with the bulk of the above cited literature to the effect that Ca does not move readily either in a downward direction from leaves or an upward direction from the roots.

Phosphorus.—Biddulph (13) has shown that downward translocation of ^{35}S and ^{32}P from the leaves of red kidney bean plants occurred principally in the phloem. He also added that the phloem of a major bundle did not function as a unit in translocation. Apparently there is no relationship between ^{32}P transport and ATP so far as the translocation of P through the phloem (petiole of primary bean leaf) is concerned [Kendall (100)].

There is conflicting evidence with regard to the presence or absence of organic P in the xylem. According to Tolbert & Wiebe (179), who examined the xylem exudate of several species of plants, three ^{32}P-labeled compounds were found, one of them being inorganic and the other two being unidentified phosphorus-containing compounds. In tomato stems, as much as 20 per cent of the ^{32}P was present in the form of the unidentified compounds. More recently, Maizel & Benson (118) found phosphoryl choline and glyceryl phosphoryl choline in alcohol extracts of barley, spinach, and *Scenedesmus.* These compounds occurred in xylem exudates and in alcoholic extracts of leaf tissue of barley after ^{32}P-labeled orthophosphate was supplied to the roots. They were also identified in the phloem exudate of leaves which had been injected with labeled orthophosphate. They concluded, therefore, that P may be translocated in plants primarily in these two forms.

Depending on the soil type and other conditions, uptake of P from the solid phase can be equal to, less than, or greater than that from solution [Islam (94)].

Sulfur.—The prevailing opinion has been that S, like Ca, is relatively immobile in plants. Immobility of both is suggested by the fact that deficiency symptoms appear on the new growth with, therefore, little or no apparent translocation of these elements from the older to the younger tissues. In a study of absorption and translocation of S in the red kidney bean plant, Biddulph *et al.* (11) demonstrated that: (*a*) pH has little effect on S uptake; (*b*) a portion of the total S within the plant remains mobile and

XUM

moves freely from one organ to another; and, (*c*) a portion of the seed S originally translocated to the roots is released for upward translocation only when S enters the plant from the root environment. Biddulph (13) concluded that S moved principally in the phloem (downward) at a rate similar to that for P and sucrose, exceeding 40 cm./hr. (11).

Tolbert & Wiebe (179) found only inorganic SO_4 in the xylem exudate of the several species of plants which they studied. Although SO_4 must be reduced before its incorporation into S-containing amino acids, proteins, and enzymes, to date nothing is known of the SO_4-reducing mechanism in plants. This report (179) and the earlier literature would indicate that SO_4 probably is reduced within individual cells since S appears to move in the conductive tissue solely as sulfate.

Iron.—Using the stable N isotope to lable the EDTA, it was revealed that in citrus, avocado, and other subtropicals, EDTA did not necessarily facilitate the translocation of Fe in plants. Wallace & North (189) therefore concluded that Fe is separated from EDTA within the plant, and that EDTA is apparently metabolized. Jones & Long (99) have shown that the ferric form of EDTA undergoes photosensitized reduction in the presence of sunlight.

DeKock (45) considered that Fe is translocated primarily in the phloem and, since the concentration of Cu is particularly high in the phloem, concluded that Cu would thus exert therein a maximum of interference. He further noted that a major part of the Fe in leaves is located in chloroplasts (attached to phosphoproteins by phosphate groups) with a small amount of the free Fe being in the ferrous state and physiologically active. This latter fraction, although low in concentration, was considered to be mobile.

Cobalt.—Because $^{60}CoCl_2$ failed to move from the leaves of bean and cucumber plants that had been in the dark for one to three days but did move when sucrose, glucose, fructose, or maltose was added, it would appear that the outward or downward movement of Co involves living cells, presumably the phloem. Glycine and mannitol were ineffective in this regard. Gustafson (74) also observed that, in the light, the absorption and translocation of ^{60}Co was increased by an increase in light intensity or temperature and the addition of S/V Sovaspray 100, but that these processes were decreased by wilting of the plants. The most recent report (75) showed that ^{60}Co entered leaves of plants kept in the dark 48 hours but that it was not translocated.

Potassium.—In view of the fact that about 30 per cent of the total K appeared to be adsorptively-bound, presumably to proteins, in beech leaves during the summer months [Olsen (148)], that fraction of the total K would presumably not be mobile. Prior to leaf fall, all of the K was observed to be in the dissolved state in the cell sap.

Strontium.—Wiebe & Kramer (200) found that ^{32}P, ^{35}S, ^{86}Rb, and ^{131}I were readily translocated upward in barley seedlings if these ions entered the roots about 30 mm. back from the tip. Ions which entered farther up the root were also readily translocated, but those that entered near the tip were

translocated little if at all. With ^{45}Ca and ^{90}Sr, however, there was little or no upward translocation regardless of point of entry. In this same connection, Menzel & Heald (129) reported that Sr is strongly held by roots.

Boron.—Because the translocation of sugar appears to be dependent on B [Gauch & Dugger (70, 71); Mitchell *et al.* (133, 134); Sisler *et al.* (164)], a discussion of the translocation of sugar inevitably involves this essential element. This relationship of B to sugars is interesting in that it implicates an inorganic constituent, boron, as affecting the distribution of an organic component, sugar. Sisler *et al.* (164) verified a role of B in sugar translocation when B-sufficient and B-deficient plants were permitted to synthesize and to translocate their own photosynthate following exposure of an intact leaf to $^{14}CO_2$.

The likelihood of sugar-boron complexes in plants is supported by the well established reaction of borate ions with OH-rich compounds such as sugars. This reaction is being currently exploited in the paper electrophoretic separation of carbohydrates. Bourne & Stephens (21) point out that by conferring a charge on the molecules of otherwise neutral carbohydrate molecules, it is possible to make separations even of polysaccharides which heretofore could not be made or could be effected only with considerable difficulty.

Previously Gauch & Dugger (70, 71) presented evidence that B is required for the translocation of sugar in higher plants, and they concluded that B-deficiency symptoms are, in reality, an expression of sugar deficiency. They postulated (70) that: (*a*) B enhanced the translocation of sugar by forming a complex with sugars which, bearing a charge (negative), traversed membranes more freely than sugar molecules, or (*b*) B might be located in the membranes and function there in the passage of sugar in a manner similar to the ion-binding compounds or carriers that have been postulated for cations and anions. Boron-sugar complexes have not been isolated from plants, and there has been no attempt to employ newly available methods and techniques in the isolation of these hypothetical compounds. The existence of B in membranes is purely speculative at the moment, though OH-rich compounds are believed to be in membranes and B is known to react with such compounds.

Recently Dugger *et al.* (47) obtained data which led them to postulate a third mechanism by which B might enhance sugar translocation. For the reaction, glucose-1-$PO_4 \rightleftharpoons$starch$+PO_4$, they observed that in the presence of B, the reaction did not proceed so rapidly to the right. Therefore, a higher concentration of soluble carbohydrates would be available (in the presence of B) at any given moment for translocation. This effect of B was determined by the leaf-infiltration technique, using glucose, and by a study of the effect of B on the starch phosphorylation reaction *in vitro*.

FUNCTIONS OR ROLES OF ELEMENTS

Introduction

It is paradoxical that the greatest advancements in our knowledge of the roles of essential elements during the last decade or so should be in the

group commonly referred to as minor or trace elements rather than the macro elements. At least one role of Mo (nitrate reduction) is well established for higher plants, and yet this element is present in only a few parts per billion in the dry matter of Mo-sufficient tomato plants.

Recent Research on Metals and Enzyme Action

An excellent review of the action of micro-nutrient elements in enzyme systems was provided by McElroy & Nason (123) in 1954. In their very complete listing of enzymes containing or requiring metals for activation, it is interesting to note that far more enzymes require Mg and Mn than any other metals. The following interrelationships of certain metals with certain enzymes have appeared since the review by McElroy & Nason (123).

Catalase.—Brown & Holmes (26) found that catalase activity was greater in Cu-deficient than in normal corn, tomato, and tobacco plants.

Indoleacetic acid oxidase (IAA oxidase).—Hillman & Galston (85) pointed out that the Mn^{++} ion has been reported to enhance, to inhibit, or to have no effect on the IAA-oxidase system of various plant breis. They observed that, with a given concentration of Mn^{++} ion in the brei, one could find either an increase or a decrease of destruction of IAA depending on the level of 2,4-D. They reasoned, therefore, that certain workers had used breis of an inherently high concentration of endogenous phenol while others had breis of a comparatively low level. Using the same concentration of Mn^{++} ion, the various workers would have found, presumably, either a stimulatory effect of Mn^{++} ion on IAA-oxidase activity or a decrease depending on the inherent level of phenol in the brei. Wagenknecht & Burris (185) presented evidence that IAA oxidase contains copper.

Adenylic kinase.—One of the few reports concerning the presence of adenylic kinase (AK) in plants is that of Mazelis (122). The enzyme catalyzes the reaction, $2ADP \rightleftharpoons ATP + AMP$. It appears to be associated with mitochondria of cotyledons from peanut and lupine seedlings and the chloroplasts of spinach and tobacco leaves. The mitochondrial enzyme seems to have a requirement for a metal with the order of effectiveness being, Mg, Mn, Co, and Ca; for the chloroplast enzyme the order is: Mg, Mn, and Co. Magnesium was clearly the best activator for both sources of the enzyme.

Aconitase.—Takeda & Hara (173) reported that a deficiency of ascorbic acid in young female guinea pigs caused a metabolism of ferrous ion, the latter being required as a co-factor for certain enzymes, particularly aconitase. They observed that the inactivation of aconitase induced a disturbance in the operation of the tricarboxylic acid cycle that was accompanied by a decrease in the activities of other enzymes such as —SH enzymes.

Xanthine oxidase.—Using ^{99}Mo, Totter *et al.* (181) showed that Mo is an integral part of the xanthine oxidase of milk and, presumably, of other sources. This finding explains one essential role of Mo in mammals. In plants, the oxidation of substrate (aldehyde or purine) can be directly linked to reduction of nitrate by xanthine oxidase [Bonner (20)].

Physiological Effects or Actions of Elements

Phosphorus.—A thorough review of the physiology and biochemistry of P in green plants was provided by Arnon (6) in 1953. In the current review certain additional aspects of P metabolism may be found in the sections on "translocation" and "chlorosis."

Magnesium ions, adenine nucleotide, and a Krebs cycle intermediate are required for the esterification of ^{32}P into $AT^{32}P$ [Mazelis & Stumpf (121)]. Formation of the latter compound is the first step in the incorporation of ^{32}P into the phospholipid fraction of mitochondria (121).

Potassium.—Bierhuizen (14) grew *Lemna minor* in nutrient solutions in which K was replaced by Na, Rb, or Cs. He reported that in the Na cultures, K deficiency caused a decrease in the rate of photosynthesis, in the chlorophyll content, the frond area and the root length, but an increase of respiration, frond dry weight, and starch content. It was observed that the increase of respiration by plants on K deficiency could be reversed by the addition of K, Rb, or Cs.

In the mechanistic role various ions play in the catalysis involving phosphate compounds, Lardy (108) stated that "one generalization which seems to be justified is that the specific requirements for divalent cations are met by rather low concentrations, while the monovalent cation (K^+) is, in many cases, required in relatively high concentration." This is an interesting generalization in view of the generally regarded "high" concentration of K that appears to be required for most plants. Certainly in view of the fact that there are no known K-containing compounds in plants, this generalization by Lardy (108) might explain the need of such a "high" concentration of K and is, therefore of interest.

In connection with evaluating relative uptakes of Rb and K by plants, basing it on changes in Rb/K ratios, Mackie & Fried (117) noted that the Rb/K ratios of leaves, nodes, and internodes of corn plants did not differ significantly but that the ratios in the reproductive parts were significantly higher than in the leaves, nodes, and internodes. The physiological significance, if any, of the higher Rb/K ratio in reproductive parts was not indicated.

The accumulation of carbohydrates has often been reported to be associated with K deficiency, and Malavolta *et al.* (119) recently made a similar observation for the cassava plant. Sugars accumulate, however, with deficiencies of many of the essential elements, presumably attributable to a disruption of the synthetic metabolism of cells. Accumulation of carbohydrates may therefore be only the indirect effect of the deficiency of a given element with no direct connection between the element and the translocation of carbohydrates per se. Many corroborative lines of direct and indirect evidence are required to establish a causal relationship between an element and the translocation of carbohydrates. In this connection, it is pertinent that Samuels & Landrau (157) reported that K increased the sugar content of cane only when it increased the yield of cane.

XUM

The physiological significance is not apparent, but the fact that Mo and K accumulate in quite different areas of leaves and stems is of interest. Stout & Meagher (170) observed that K accumulated in the veins of leaves and in stems, whereas Mo accumulated in interveinal areas and did not accumulate in the stem.

Nitrogen.—A symposium on inorganic N metabolism, with special emphasis on metallo-flavoproteins, has recently been published [McElroy & Glass, Eds. (124)]. The involvement of Mo in N metabolism, by way of NO_3 reduction, is thoroughly stressed in this important contribution (see also "molybdenum" in this section).

The reduction of NO_3, via reduced coenzymes I and II, does not necessarily depend on the oxidation of carbohydrate intermediates [Evans & Nason (58)]. Nitrate may be reduced through a photochemical reduction, involving the following reactions:

$$H_2O + TPN \xrightarrow{\text{(light \& grana)}} \tfrac{1}{2}O_2 + TPNH + H^+$$

$$TPNH + H^+ + NO_3^- \xrightarrow{\text{(nitrate reductase)}} NO_2^- + TPN + H_2O$$

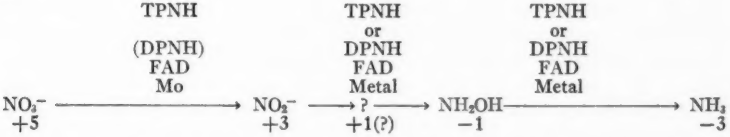
The concentration of NO_3^- (0.02 *M*) that was optimal for NO_3 reductase activity resulted in an approximately 75 per cent inhibition of the photochemical reduction of TPN (58). They pointed out that this inhibitory effect of NO_3 reduction on the photochemical reduction of TPN might explain reports in the literature to the effect that N-deficient plants are more active photosynthetically than leaves of comparable plants provided with adequate nitrate. Nason *et al.* (137) obtained a partially purified nitrite reductase from *Neurospora;* the enzyme required FAD and a metal component.

Florell (63) concluded that Ca favored the formation of mitochondria. An external calcium concentration of 10^{-4} *M* increased the mitochondria by 54 per cent and their protein content by 29 per cent, as compared with controls. He suggested that this effect of Ca on mitochondria might explain Burstrom's (34) observation concerning an enhancement of nitrate uptake by calcium.

The original observation by Hewitt & Jones (82) that NO_3 accumulates in Mo-deficient plants has been verified by Wilson & Waring (202) and most recently by Steinberg (168) using tobacco. As had been shown earlier for *Chlorella pyrenoidosa* by Walker (186), the requirement of *Scenedesmus* for Mo could also be overcome by supplying the cells with urea or ammonia [Ichioka & Arnon (93)]. In this alga, the function of Mo was found to be limited to NO_3 reduction—apparently the first instance in the nutrition of green plants in which the essentiality of an inorganic element was linked with only one enzyme system.

McElroy & Spencer (125) pointed out recently that the literature supports the view that there are at least three different types of NO_3-reducing systems. With regard to the process of NO_3 assimilation, they state that there are two broad pathways, viz., an inorganic pathway involving the stepwise

reduction of NO_3 by a series of two-electron transfers, until ammonia is produced, at which stage it is incorporated into an organic molecule; or, the incorporation of inorganic N into an organic molecule at an oxidation level higher than that of ammonia, with this compound then being reduced to the amino group level. Nason (138) summarized, as follows, the pathway with regard to participating agents and the oxidative steps of the various forms of N:



Vanecko & Varner (184) presented evidence that NO_2 is an intermediate in NO_3 reduction. This observation does not, of course, preclude the possibility of direct incorporation of N into an organic molecule with reduction to the amino group level occurring later (125). Zucker & Nason (205) isolated hydroxylamine reductase from *Neurospora*, and partially purified it. They pointed out that hydroxylamine has long been considered an intermediate in the biological reduction of NO_3 to NO_2 to ammonia. Nicholas & Nason (141) presented evidence that, during the transfer of electrons from TPNH to NO_3, both FAD (or FMN) and Mo function as electron carriers. Later work by Nicholas & Nason (143) showed that reduced FMN or Mo could replace TPNH in the reduction of NO_3 to nitrite.

According to Watanabe (195) it is surprising how few algae excrete amino acids into the external, bathing medium. Only *Calothrix brevissima* excreted amino acids—aspartic, glutamic, and alanine. Essentially no nitrogen was excreted by *Tolypothrix tenuis*, *Anabaeopsis* sp., and *Nostoc* sp. It is possible that the appropriateness of a given regime of mineral nutrition and other factors may determine whether or not N compounds will be excreted. Fogg (64) observed that under certain conditions, as much as 30 to 40 per cent of the N fixed by *Anabaena cylindrica* appeared in the medium in soluble form. Iron and K deficiencies were associated with the appearance of extracellular N in the medium whereas, surprisingly, Mo deficiency was not associated with a loss of N to the medium. As was shown by putting algal cells into a N-deficient medium, Krauss (103) reported that "equilibration is strongly toward protein synthesis" and that "even under conditions of carbohydrate deficiency in the dark, only a slight increase in proteolysis was observed and little or no deamination could be detected." Fogg (65) has recently published a comprehensive review of N fixation by photosynthetic organisms.

Sulfur.—The absorption and translocation of S have been covered under those two respective headings in earlier sections. Briefly summarized: (*a*) pH was reported to have little effect on the absorption of S; (*b*) a portion of the S remains mobile in the plant; (*c*) seed S translocated to the roots is not

XUM

free to move upward again unless SO_4 is entering the roots; and, (*d*) the downward movement of S occurs in the phloem moving at a rate commensurate with that of P and sucrose, viz., 40 cm./hr. [Biddulph *et al.* (11); Biddulph (13)]. Leggett & Epstein (109) reported, however, that pH has an effect on SO_4 absorption with the highest absorption rate occurring at pH 4. They also showed that Ca enhanced the absorption of SO_4 with the maximal activation being 203 per cent that of the control plants. Calcium may be assumed to increase the turn-over rate of the sulfate-carrier complex.

Sulfate and SeO_4 have been shown to compete for site during absorption [Leggett & Epstein (109); Weissman & Trelease (198)]. Weissman & Trelease (198) noted that added S-amino acids appeared to overcome SeO_4 inhibition in *Aspergillus niger* by competing successfully with Se analogues. This hypothesis was supported by data which indicated that absorbed SeO_4 was converted to organic forms in the absence of added S-amino acids, but that SeO_4 tended to remain in the inorganic form in the presence of methionine. Confirming the prevailing opinion on the subject, Tolbert & Wiebe (179) found S to be present in only the SO_4 form in the xylem exudate of several species of plants. Despite the fact that S must be reduced prior to its incorporation into organic combination, to date literally nothing is known of the location or the mechanism of SO_4 reduction in plants.

Calcium.—Certain aspects of the absorption and translocation of Ca were treated earlier under those topic designations. The current section is thus reserved primarily for physiological effects or roles of Ca.

Allen & Arnon (1) reported that, for certain N-fixing blue-green algae, Ca was essential and irreplaceable for growth regardless of whether NO_3 or N_2 was supplied. Epstein & Leggett (53) reported that Ca, Sr, and Ba compete for identical binding sites or reactive centers on the carriers and that Mg passes through membranes over a separate site. Florell (63) obtained evidence that Ca favored the formation of and increased the protein content of mitochondria. In view of the role of mitochondria in aerobic respiration and hence salt uptake, a direct relationship between Ca and the uptake of ions in general is indicated.

Recent evidence has been presented by Geraldson (72) that blossom end rot of tomatoes is caused by a localized deficiency of Ca at the blossom end of the fruit. He explained the "apparent" relationship of soil moisture to Ca deficiency as being attributable to changes in Ca availability and absorption. He proposed that during the day Ca may move out of the leaves in the form of Ca gluconate.

Inasmuch as Ca has been shown to increase the rate of SO_4 [Leggett & Epstein (109)] and HPO_4 absorption [Leggett (110)] by excised barley roots, apparently by increasing the turn-over rate of the SO_4 and HPO_4 carrier complexes, this interesting effect of Ca may be shown to affect similarly the absorption of other ions. In fact, Tanada (174) has recently reported that the uptake of Rb and P were enhanced by the presence of calcium.

The stimulatory effect of Ca on K absorption was explained by Nielsen & Overstreet (144) as being the result of Ca acting as a co-factor in the utiliza-

tion of the K-carrying complex—an event which would tend to enhance the reaction of K with the carrier. An explanation was not offered, in terms of their concept, to explain the earlier report by Overstreet *et al.* (150) that Ca may increase or decrease K absorption depending on the level of potassium. The presence of an additional ion, viz., Ca^{++}, would have been expected to decrease K absorption, whereas actually Ca increased it (150). It is conceivable, however, that at other proportionalities of K and Ca, Ca could decrease K absorption.

Ca has been shown to act as a co-factor for AK obtained from the mitochondria of peanut and lupine seedlings. It was not as effective, however, as the Mg^{++} ion [Mazelis (122)].

Although not of recent date, the earlier observation by Skok (165) is extremely interesting with regard to a possible interrelationship between Ca and N metabolism. Confirming earlier work by Gauch (69) and Nightingale *et al.* (145), he observed that −Ca plants were unable to absorb or utilize NO_3; that they made little growth, had pronounced Ca-deficiency symptoms, and died very early. Burström (34) also found that Ca was required for NO_3 uptake. Gauch (69) showed that −Ca plants could, however, absorb appreciable quantities of NO_3 if the Mg level of the solution were sufficiently reduced as compared with the level used by Nightingale *et al.* (145). Even though NO_3 was then absorbed, the plants made little growth and Ca-deficiency symptoms were only slightly delayed. Skok (165) showed that if urea was supplied to −Ca plants instead of NO_3 that the plants weighed 60 per cent more, that Ca-deficiency symptoms were much delayed, and that when the symptoms became evident, they were much less severe. Roots of such plants did not exhibit the usual Ca-deficiency symptoms and produced twice as much growth as those of −Ca plants supplied with nitrate. Similarly, Bolle-Jones (19) noted that the Ca requirement of *Hevea brasiliensis* was much decreased if ammonium salts were used as a source of N rather than nitrate. Gauch (69) observed that −Ca plants were unable to mobilize and use the Ca of their cotyledons. Ironically, plants which were receiving Ca in the external solution were also able to mobilize and translocate the Ca of their cotyledons. It would be interesting to determine whether nutrition with urea-N would promote a mobilization and utilization of Ca from the cotyledons of −Ca plants.

There are, as yet, certain unexplained differences in the capacities of different plants to remove Ca from a given soil. Walker *et al.* (188) noted that plants which are native to serpentine soils are much more efficient at removing the characteristically low amount of Ca from these soils than are those species which are not indigenous to serpentine soils. A difference in the concentrations or activities of Ca and Mg ion-binding sites in the roots could be a factor.

Although not of recent origin, the research by Conrad (36) appears to merit special consideration in view of the continuing question concerning the chemical nature of the middle lamella and in view of the fact that Conrad's findings have so frequently been overlooked. He examined 14 types of

XUM

plant materials and only in pithy radishes was any "pectate" (determined as pectic acid) found. In that tissue it represented 15.37 per cent of the dry weight. On the other hand, when parsnip tissue, for example, was dried at 98°C. in a hot-air oven, at 70°C. in vacuum, sun-dried, or put directly into boiling alchol, Conrad found 3.95, 9.61, 5.26, and 0.00 per cent (dry weight basis) of pectic acid. The absence of pectate in 13 out of 14 plant tissues placed directly into boiling alcohol would strongly indicate that there is no pectate normally in most plant tissues and that it is formed only during the heating and drying process. If there is indeed no pectic acid, there can obviously be no Ca-pectate in the middle lamella or otherwise. Presumably, then, in most plants the pectic enzymes become very active during the heating and drying of the tissue with the inevitable result that Ca- and Mg-pectates are formed as "artifacts." In Kertesz's (101) monumental discussion of the pectic substances, he recognizes the significance of Conrad's (36) findings and therefore stressed the importance of rapid inactivation of the pectin-destroying enzymes in the preservation of plant material for pectic analyses. Bolhuis & Stubbs (17) recently stated that Ca is a constituent of the middle lamella but did not specify that the latter is composed of Ca-pectate.

Drosdorff *et al.* (46) claim that Ca and Mg may function interchangeably in such functions as precipitation of oxalate anions. They also noted for tung trees and at low levels of either Ca or Mg, that Mg and Ca, respectively, could stubstitute for the other slightly-deficient element.

Burström (34) found that Ca exerted two effects not produced by K, Fe, or B, i.e., Ca increased the elasticity of the cell walls but was dispensable for the effect on plasticity. He concluded that growth of cells starts with an increase in the tensibility of the walls, which is independent of Ca, and that this is followed by a Ca-induced formation of the elastic components of the final wall by intussusception.

Calcium is considered a micronutrient for most algae, including *C. pyrenoidosa* [Walker (187)]. He reported that only 10 μg. of Ca were required per gm. dry weight of algae as compared with 30 μg. for Fe. For this organism, an equimolar concentration of Sr could be substituted for the Ca. According to Walker (187), algae differ with respect to their responses to Sr. *Scenedesmus obliquus*, strains D-1, and D-3, and *Selenastrum minutum* could not have their requirement for Ca substituted by Sr. The growth of *Coccomyxa pringsheimii* was strongly inhibited by Sr. Brusca & Haas (31) observed that the small leaves produced by Zn-deficiency in citrus would become green if Zn were supplied but that they would not also enlarge unless there was adequate calcium.

Iron.—The relationship of Fe to Cu is discussed in the section on "interrelationships of ions" and the relationship of Fe to chlorosis in the section on "chlorosis."

With regard to Fe and compositional changes, Brown & Wadleigh (29) noted that the HCO_3^- ion resulted in an increase in accumulation of monovalent ions in leaves—Na or K, depending on specificities of the plants. They postulated that this increase in Na plus K or in the ratio of monovalent

to diavalent cations might affect the activity of Fe, possibly through the precipitation of Fe phosphates. Treatment and chlorosis were not correlated with any particular cation or with the K/Ca ratio in bean and beet plants but rather with monovalent cations or the Na+K/Ca+Mg ratio.

According to DeKock (45), the major part of Fe in leaves is located in chloroplasts, attached therein by phosphate groups to phosphoproteins with only a small amount of free Fe being in the ferrous state and physiologically active. He further concluded that Fe controls the amount of phosphoprotein and the concentration of other heavy metals associated with the phosphoproteins. Both Sideris & Young (163) and Jacobson & Oertli (96) have stated that Fe is involved primarily in the formation of chloroplastic protein in leaves, directly or indirectly. As might be expected, N has also been shown to be required (163) for the formation of chloroplastic protein.

Working with ascorbic acid deficiency in guinea pigs, Takeda & Hara (173) claimed that ascorbic acid deficiency resulted in a derangement of the metabolism of ferrous ion causing an inactivation of the enzymes which require the ferrous ion as a co-factor, such as aconitase. The inactivation of aconitase supposedly induced a disturbance in the operation of the tricarboxylic acid cycle, and this was accompanied by a decrease in the activities of other enzymes such as −SH enzymes. There would appear to be merit in determining to what extent alterations in ferrous Fe in plants might lead to a similar series of events in plants. In this connection, Thimann (176) has recently stated that the growth inhibition caused by excess of Co was probably attributable to inactivation of a sulfhydryl group. It might perhaps be worthwhile examining the possibility that this inactivation was caused by a cobalt-induced alteration in ferrous ion level.

Haertl & Martell (77) recently discussed in detail the chemistry of and the role of metal chelates, including Fe-chelate, with regard to plant and animal nutrition. Krauss (104) reported favorably on the use of chelated metals in continuous, mass cultures of algae and he recently discussed certain fundamental reactions of chelating compounds (105). He stressed that there must be localized modification of chelating efficiency with various metals since Mg, which is held only weakly by strained bond angles in the porphyrin chlorophyll molecule, is not readily displaced by Cu or Fe. Certain relatively new chelates have been reported to be more effective for preventing chlorosis in alkaline calcareous soils [Holmes & Brown (86); Wallace *et al.* (190)]. The latter authors also reported that, with certain new chelates, there was less fixation on the clay fraction in soil and relatively less toxicity than with other chelating agents. Although Mn, B, and possibly the Zn requirements of plants could be satisfied by high concentrations of fritted trace elements or "glassy frits," this type of material was found unsatisfactory as a source of iron for plants [Rhoads *et al.* (153)].

Magnesium.—One of the more recently studied roles of Mg involves its participation, along with adenine nucleotide and a Krebs cycle intermediate, in the esterification of P into ATP [Mazelis & Stumpf (121)]. This is the first step in the incorporation of P into the lipid-P fraction of mitochondria.

XUM

Another recently studied action of Mg is its role as a co-factor for the action of AK which catalyzes the reaction, $2ADP \rightleftharpoons ATP + AMP$. For both the AK associated with mitochondria and that associated with chloroplasts, Mg was the most active and efficient co-factor although certain other metals were active to a lesser degree [Mazelis (122)]. The interrelationships of Mg to Ca and to P are discussed under "interrelationships of elements."

Manganese.—Anderson & Evans (3) found that physiological levels of Mn had no effect on the concentration or activity of isocitric dehydrogenase and "malic" enzyme, but that Mn toxicity increased the activity two to three times. The variable effect of Mn on IAA oxidase, reported in the literature, was explained in terms of variation in endogenous levels of 2,4-dichlorophenol [Hillman & Galston (85)]. Depending on the endogenous level of phenol—assuming a constant concentration of Mn—one could obtain an increase, a decrease, or no effect of Mn on IAA oxidase. Mn may substitute to a less effective degree for Mg as a co-factor for the action of AK, according to Mazelis (122).

Zinc.—There appears to have been no additional research on the direct role of Zn in the formation of tryptophane, from whence IAA is supposedly formed in plants. The role of Zn in the reproductive processes of the corn plant has received added confirmation and elaboration by the work of Lingle & Holmberg (113). With regard to the absorption of Zn from soil, Wear (196) presented evidence that the pH—and not Ca—affects Zn uptake.

Wallihan & Heymann-Herschberg (193) noted that, in citrus, Zn absorption and translocation occurred more rapidly when applied to young than to old leaves. Both processes were enhanced by applying the Zn near the center of the leaf rather than near the edge. Inasmuch as equal rates of absorption and translocation occurred whether the Zn was applied to the upper or to the lower surface, entry of the Zn through stomata was slight or did not occur. With Zn-sufficient plants, the ultimate distribution of the applied Zn was the same whether it was absorbed by the leaves or the roots. Although there was no suggestion as to the significance of the observation, Ozanne (151) noted that increases in light intensity were associated with decreases in the concentration of Zn in the leaves of subterranean clover.

Copper.—The enzymes known to contain Cu include the oxidases of ascorbic acid, *p*-phenylenediamine, and polyphenols such as tyrosine [Glass (73)]. The possible role of Cu in respiration is clearly indicated. Copper may also prove to be at least one of the metals concerned with the light reaction of photosynthesis in green plants (73). Wagenknecht & Burris (185) presented evidence that IAA oxidase also contains copper. In view of the well known accumulation of Fe in the nodes of K-deficient corn plants, it is pertinent that, according to Brown & Holmes (26), a deficiency of Cu may also cause an accumulation of Fe in corn plants, especially at the nodes. Arakatsu & Ashida (4) found that the addition of certain amino acids to the medium prevented toxicity to copper of one strain of *Saccharomyces ellipsoideus.* Apparently a Cu-resistant strain of this organism owed its immunity to its ability to synthesize glutamic acid in the presence of copper.

Boron.—Additional information is available concerning the effect of B on the water relations of plants. Baker *et al.* (10) observed that the transpiration rate of −B plants was less than that of boron-sufficient bean plants. The reduction in transpiration was attributed to three factors: (*a*) a decreased rate of water absorption by −B plants (based on results in the literature); (*b*) the fact that the leaves of −B plants had an abnormal leaf morphology with a high percentage of abnormal and apparently nonfunctional stomates (with only one guard cell); and (*c*) a higher sugar and hydrophilic colloid concentration. The difference in hydrophilic colloids of −B and +B plants was indicated by the respective capacities of dry tissues to rehydrate. The difference in hydration—presumably because of hydrophilic colloids—was largely accounted for in the −B plants by increased concentrations of pectins and possibly pentosans.

An abnormal condition in the growth of raspberries, known as "dieback," was shown by Monk (135) to be associated with B deficiency. He stated that the "most characteristic leaf symptom of boron deficiency in raspberry is the 'feathery' appearance where the leaves are elongated, thin, and deeply indented." According to the pictures of the deficiency, this reviewer is impressed with the close similarity between the "feathery" appearance of the B-deficient raspberry (135) leaves and the "feathery" appearance of leaves of certain plants that have received 2,4-D. Cripps (37) reported a foliar abnormality in hop plants associated with B deficiency and referred to as "ivy leaf."

Apparently related to the fact that B deficiency causes excessive root branching and because of the fact that the site of nicotine synthesis in tobacco is in the apices of shoot and root, B deficiency caused an increase in the synthesis of nicotine [Steinberg (167)].

Sisler *et al.* (164) verified a role of B in the translocation of sugars. Dugger *et al.* (47) obtained data with respect to the action of B on starch phosphorylase that provides an additional explanation as to how B might affect the translocation of sugars (see section on "Translocation—Boron"). Confirming an earlier report by Sisler *et al.* (164), McIlrath *et al.* (126) were unable to prevent or overcome B-deficiency symptoms by spraying the tops of −B plants with sugar. Plants which were sprayed with sugar and B grew almost as satisfactorily as plants receiving B in the nutrient solution. Although it was not tried, it is possible that −B plants would have grown nearly as well and would have been nearly as free of deficiency symptoms as those receiving B in the nutrient if they had received a foliar application of B alone.

In a study of the translocation of ^{14}C-labeled sugars in soybean leaves, Nelson (139) noted that the addition of B to a detergent greatly enhanced the absorption and translocation of sugar as compared with the entry of sugars applied alone or in the presence of detergent. McElroy & Glass (124) pointed out that Mo, as well as B, has the capacity to react with sugar alcohols as well as with hydroxy acids.

Molybdenum.—Mo deficiency has been reported from 16 states of the

XUM

United States, with the most extensive area involving the citrus region of Florida [Rubins (156)]. The March, 1956, issue of *Soil Science* was devoted entirely to various aspects of Mo in soil, plants, and animals. Therein Hewitt (83) reviewed the deficiency symptoms of many kinds of plants. Apparently Hewitt (82) was among the first—if not the first—to report that NO_3 accumulates in Mo-deficient plants. This accumulation of NO_3 has been reported by others, among the more recent ones, Stout & Meagher (170), Rubins (155), Steinberg (168), Arnon *et al.* (8), and McElroy & Glass (124). In 1954 and 1955 Nicholas & Nason showed that the nitrate reductases from *Neurospora* (140) and *Escherichia coli* (142) were specifically activated by Mo, and in 1955 Evans & Hall (59) demonstrated that Mo is an actual constituent of soybean nitrate reductase. The latter added that Fe may partially replace Mo as a metal co-factor for this last reductase. Shug *et al.* (162) have shown an association of Mo with hydrogenase in the aerobic N-fixing bacterium, *Clostridium pasteurianum.* Evans (60) also indicated that Mo is required for N fixation. The role of Mo as an electron carrier in NO_3 reduction is explained in some detail in an earlier section on "Physiological Effects or Actions of Elements—Nitrogen."

Several authors have reported that plants do not require Mo if given a reduced form of nitrogen. This has most recently been reported for *A. niger* [Mulder (136)], *Chlorella* [Walker (186)], and *Scenedesmus* [Ichioka & Arnon (93)]. In *Scenedesmus,* Mo apparently played a role only in NO_3 reduction and was not required for any other role (93). Arnon *et al.* (8) and Evans (60) have pointed out that for many plants there appears to be a need for Mo beyond that for NO_3 reduction since Mo proved beneficial even when a reduced form of N was supplied. Allen & Arnon (1) recently reported that this was so for *A. cylindrica.*

The disappearance of chlorosis when reduced forms of N were supplied to Mo-deficient cultures of *Scenedesmus* [Ichioka & Arnon (93)] was interpreted to indicate that the observed effect of Mo on chlorophyll synthesis was indirect and that it acted only when NO_3 reduction was blocked. If this is true, deficiencies of various essential elements results in NO_3 accumulation and chlorosis, and it may be that certain of these deficiencies, too, are also only indirectly and not causally related to chlorosis. It would appear to be worthwhile to determine whether certain of these other element-deficiency-induced chloroses would disappear if reduced N were supplied.

Loneragan & Arnon (115) noted that for *Chlorella,* Mo deficiency had little effect on the dry weight of cells, doubled the respiration, and reduced chlorophyll content and photosynthesis to one-third that of Mo-sufficient cells (those receiving Mo at a concentration of 10 p.p.b.). The depression in photosynthesis was correlated with the reduction in chlorophyll.

Very small amounts of Mo overcome deficiencies of the element under field conditions. In Australia, 24 ounces of molybdic oxide are mixed with 2,240 lb. of superphosphate [Anderson (2)] prior to application of the superphosphate. Arnon *et al.* (8) have determined that 3,000 atoms of Mo per cell

give good growth of *Scenedesmus;* cell division failed when there were only 1,500 to 1,700 atoms of Mo per cell. Mo-sufficient tomato plants have as little as 10 parts of Mo per billion parts of green weight [Stout & Meagher (170)].

For the effect of Mo on xanthine oxidase, see earlier section, "Recent Research on Metals and Enzyme Action—Xanthine oxidase."

Sodium.—During ion absorption, Na does not compete for site with K, Rb, or Cs which are competitive with each other [Epstein & Hagen (49)]. Scott & Hayward (158) confirmed the independence of movement of Na and K in *Ulva lactuca.* Takada (172) has reported similarly that Na does not interfere competitively with K in strand plants. With regard to the essentiality of Na, Kratz & Myers (102) showed that both Na and K are required for maintenance of maximal growth rate of *Anabaena variabilis, Anacystis nidulans,* and a strain of *Nostoc muscorum.* For cotton, Selman & Rouse (160) noted that the addition of Na to the nutrient solution increased the early fruiting and boll maturity of cotton grown under conditions of poor aeration but not under conditions of good aeration. Early fruiting, however, did not affect the final yield, and only when K was deficient was a yield response to Na obtained. Similarly, working with artificial culture conditions, Ulrich & Ohki (183) reported that Na increased the growth of low K sugar beet plants but had no effect on high K plants.

Lithium, rubidium, and cesium.—K, Rb, or Cs were shown to reverse the enhanced respiration of K-deficient *L. minor* [Bierhuizen (14)]. K, Rb, and Cs interfere competitively with each other in absorption, thus indicating that these ions compete for identical binding sites. Na and Li do not compete with these ions for site [Epstein & Hagen (49)]. Cs has been reported to have only about one-fifth the specific rate of entry of K into the roots of plants [Menzel & Heald (129)], thus indicating that the site for K, Rb, and Cs has a greater affinity for K than for Cs.

Strontium and barium.—Ca, Ba, and Sr interfere competitively in absorption, whereas Mg enters over a separate site [Epstein & Leggett (53)]. The Ca requirement of *A. cylindrica* could not be replaced by Sr [Allen & Arnon (1)]. Bowen & Dymond (22) observed that Sr was preferentially absorbed with respect to Ca from most soils, while Ba was taken up less readily. Native plants were found to contain concentrations of Sr up to 2.6 per cent of the dry weight when grown on Sr-rich soils. Such an accumulation is striking in any event but it is particularly striking in view of the fact that Menzel & Heald (129) reported that Sr was strongly held by roots.

Bromine, chlorine, and iodine.—Chloride has been shown to compete with Br in absorption, thus indicating identical binding sites; NO_3 did not compete with Br for site [Epstein (50)]. Stout *et al.* (171) have recently shown that the element, Cl, is essential for the growth and reproduction of tomato. The addition of Br only slightly delayed Cl-deficiency symptoms.

Nereocystis luektkeana has been found to concentrate I to the extent of 6 to 10 times that of the nutrient solution, with I being present in both

XUM

inorganic and organic forms. The organic I was shown to yield principally mono- and di-iodotyrosine upon hydrolysis [Tong & Chaikoff (180)]. It is interesting that these iodine-containing compounds were isolated from algae before their significance in the thyroid gland was elucidated.

Chlorine deficiency symptoms of sugar beet have been reported to show first on young leaves and, by transmitted light, to resemble Mn-deficiency symptoms [Ulrich & Ohki (183)]. Later, however, the interveinal areas appeared as smooth, flat depressions, light green to yellow in color, which was in striking contrast to the green veins having a "raised" appearance. They stated that the advanced stages of Cl deficiency were unique and clearly distinguishable from those of Mn, Fe, or Mo. The roots had a very stubby growth, with many stubs arising from secondary roots to form a distinctive and abnormal root structure. "Chloride stimulation" was observed in that the fresh weight of tops and roots of plants receiving 3 m.eq. Cl/l. was around 50 per cent greater than that of plants receiving 0.0006 m.eq. Cl/l. They also reported that Br prevented Cl deficiency in low-Cl solutions and that the growth of tops and roots was excellent. In view of the apparent complete replacement of the need for Cl by Br, one of the criteria for "essentiality" would appear to be unsatisfied with respect to chlorine. They further added that Cl was "necessary for top and root growth and was associated with sugar formation rather than sugar utilization." This observation is of interest in connection with Arnon's (5) suggestion that Cl may be a coenzyme in photosynthesis.

Aluminum, selenium, nickel, and vanadium.—Aluminum remains of interest in plant nutrition and has been reported to be an activator of ascorbic acid oxidase [McElroy & Nason (123)]. Selenate interferes competitively with SO_4 in absorption, whereas PO_4, NO_3, and Cl showed no measurable affinity for the SO_4-SeO_4 binding site, according to Leggett & Epstein (109). Nickel is of interest in that there is evidence that it is required by enolphosphopyruvic adenosinediphosphate transphosphorylase [McElroy & Nason (123)]. Vanadium was shown to be unacceptable as a substitute for Mo in the nutrition of *A. cylindrica* [Allen & Arnon (1)]. Vanadium was, however, found to be essential and hence irreplaceable for *S. obliquus* [Arnon & Wessel (7)].

Cobalt.—There is evidence that the cobaltous ion may somehow depress the rate of peroxide genesis or that it may aid in the decomposition of peroxides as they are formed and before they are utilized in peroxidative reactions [Galston & Siegel (68)]. Such Co-initiated chain decompositions of hydroperoxides have been reported. Inasmuch as Co mimics the effect of red light in promoting the growth of certain plant cells, there is the suggestion (68) that the morphogenic action of red light may be associated with peroxide metabolism, perhaps through the IAA-oxidase system. $Cobalt^{++}$ ion reduced apparent peroxidgenesis, curtailed IAA destruction, and prevented O_2 and x-ray damage. Galston & Siegel (68) suggested that decreased peroxide levels could account for the prevention of injury.

Certain actions of Co are reminiscent of the action of hormones. Thimann (176) noted that Co increased the elongation of etiolated pea stems in auxin and that its effect was almost doubled in the presence of sucrose. Ni had a smaller effect and Mn still less; Fe inhibited this response as well as an effect on curvature of split pea stem sections. Co apparently did not act via the formation of vitamin B_{12}, nor was the action associated with increased salt uptake. It was concluded that Co promoted some step in oxidative metabolism which normally makes a source of energy (perhaps ATP) available for growth, and divorced it from other metabolic roles. He obtained evidence that Co may activate an ATP-using process which thus deprives the plant of the means by which it can utilize acetate. In this connection, Co acts much like auxin. Tissues which were rich in acetate but poor in sugar were inhibited, while those which were poor in acetate and rich in sugar were promoted. This would be like auxin in that the same growth factor, viz., Mo, in the same concentration, would elicit opposite responses in different tissues. This is (176) precisely the classical paradox of the inhibition of lateral buds by auxin with concentrations which promote elongation of the stem.

Holm-Hansen *et al.* (87) found that Co was essential for *N. muscorum*, *Calothrix parietina*, *Coccochloris peniocytis*, and *Diplocystis aeruginosa*. Inasmuch as the Co requirement could be satisfied by the addition of relatively minute amounts of vitamin B_{12}, there was the suggestion that vitamin B_{12} was required by these organisms and that Co was required solely because it is a constituent of this vitamin. They pointed out that Co has not been demonstrated to be essential for higher plants, and that reports of B_{12} in higher plants may be related to the occurrence of associated organisms containing vitamin B_{12}.

While studying the metabolism of ^{60}Co by *Rhodymenia palmata*, Scott & Ericson (159) observed that the ^{60}Co was not found in vitamin B_{12} of these organisms. They concluded that this organism, and perhaps other marine algae, do not manufacture the vitamin but, rather, that they absorb it from the medium, with bacteria having produced the vitamin. Cobalt has been found to substitute, although less effectively, for Mg as an activator of AK [Mazelis (122)].

Silicon.—Although Ulrich & Ohki (183) obtained inconclusive results with regard to Si in sugar beets, Lewin (111) showed clearly that the diatom, *N. pelliculosa* (Breb.) Hilse had an obligate requirement for Si. A concentration of 35 p.p.m. gave the best growth. Lewin (112) also reported that Si uptake was linked with aerobic respiration and that respiration was increased when Si was supplied to Si-deficient cells of this diatom.

INTERRELATIONSHIPS OF ELEMENTS

In this section are covered those interrelationships of ions other than those very specifically associated with chlorosis.

Boll (18) noted that the growth of excised tomato roots with 0.01 p.p.m. of Mo, in the presence of 0.001 p.p.m. of Cu, was equal to or less than the

XUM

control with no microelements. However, the same concentration of Mo produced a marked increase in growth in the absence of Cu, thus indicating a possible existence of an "antagonistic" effect between Cu and Mo.

Low concentrations of Ca in the culture solution stimulated K absorption by barley roots in the pH range of 2 to 6 [Fawzy *et al.* (61)]. Earlier, however, Overstreet *et al.* (150) emphasized that Ca can increase or decrease K absorption depending on the concentration of potassium. Recently Nielsen & Overstreet (144) showed that Ca enhances K absorption and they postulated that Ca functioned as a cofactor in the utilization of the K carrier. Joham (97) reported that Na had no effect on Ca absorption from −Ca solutions in which cotton was grown, but that Na prevented shedding of older leaves under −Ca conditions. He postulated that Ca of the older leaves may have been available for translocation to the bolls. Na had no effect on K deficiency symptoms, total growth, and absorption of K in −K solutions in which cotton was grown, but Na greatly improved fruiting under K and Ca deficiencies [Joham (97)]. For *Scenedesmus* under conditions of K deficiency, Thomas and Krauss (178) noted that Na accumulated in proportion to the decrease in K; that when K was again supplied that Na dropped in direct proportion in the cells to the extent to which K increased. With regard to absorption, Na and K have been shown not to compete, so the changes which Thomas & Krauss (178), and others, have reported must be attributable to internal control by the cells. Haas & Brusca (76) found that with a constant external K level, increasing external concentrations of Na increased the K in the leaves, stems, and roots of citrus; Na decreased Ca in the leaves and roots.

The relationship between Mg and P has been elucidated by Mazelis & Stumpf (121) who showed that Mg^{++} ions, adenine nucleotide, and a Krebs cycle intermediate were required for the incorporation of P into ATP. Using synthetic ion-exchange resins, Wallace & Ashcroft (191) found that the Mg level did not influence the concentration of P in rough lemon, avocado, barley, and bush beans. Magnesium did increase the P level in certain crops with a low Mg requirement when P was present in a relatively high concentration externally.

Although previous work has demonstrated that B has little effect on the total Ca in plants, McIlrath & DeBruyn (127) noted that in Siberian millet increasing external concentrations of B increased both the percentage of soluble and total Ca in the plants. In sand culture, Cripps (37) noted that high external concentrations of Ca accentuated B-deficiency symptoms in hop plants.

With regard to the Mn/Cu relationship, Steinberg *et al.* (168) observed that Mn deficiency was associated with a 5- to 6-fold increase in the Cu concentration of tobacco plants. Löhnis (114) observed that there was a Mn/Mg antagonism in certain plants; that it was absent in others. Zn deficiency in tobacco was associated with a 2- to 3-fold increase of Mn in tobacco plants [Steinberg *et al.* (168)]. In addition to the selective absorption of Ca and Mg

by plants, Walker *et al.* (188) noted that plants indigenous to serpentine soils, of characteristically low Ca, absorbed more Ca than plants not native to this soil type.

Warington (194) obtained data which indicated a very definite interrelationship between Fe and Mo. Although high amounts of Mo were required, Mo mitigated both Fe deficiency and excess. Evans & Hall (59) showed that Fe may partially replace Mo as the metal cofactor for soybean nitrate reductase, thus indicating the nature of one relationship between Fe and Mo. Jones (98) discussed evidence which indicates that the Mo concentration in plants may be related to both pH and the concentration of Fe in soils. In ferruginous soils Mo is held on the surface of colloidal ferric oxides as the molybdate anion and is replaceable by OH^- ion.

Working with oat plants, Crooke (39) noted that there was a linear relationship between the degree of leaf necrosis and the Ni/Fe ratio in the plants. Increased Fe reduced Ni uptake, and vice versa. With regard to a Ca/Zn effect, Wear (196) has recently presented evidence that Zn absorption by plants is a function of pH and not of Ca concentration in the soil. Stout & Meagher (170) obtained clearcut evidence that there was a Mo/P relationship. As the concentration of phosphate ions in the nutrient solution was increased, Mo uptake was increased. Cain (35) reported that, for apple seedlings in sand cultures neither K nor Mg influenced the rate of absorption of the other elements. These two cations have been shown, by other workers, not to compete for site on carriers. The Fe/P interrelationship is discussed in the following section.

CHLOROSIS

Inasmuch as most of the recent research suggests that the Fe/P relationship is a cardinal one in chlorosis, factors other than this relationship will first be presented, followed by a discussion of the significance of the Fe/P ratio in chlorosis.

Jacobson & Oertli (96) stated that Fe chlorosis is initially caused by a simple deficiency of iron. They postulated that a deficiency of Fe progressively impairs the chlorophyll-producing mechanism to the point that chlorophyll synthesis is limited not by the amount of Fe in the leaf but the inability of the leaf to produce chlorophyll. According to this view, it is not the Fe which becomes inactive but rather the leaf becomes inactive because of a period of Fe shortage. They observed, for example, a good correlation between Fe and chlorophyll content of plants on a continuous supply of Fe, but no relationship on an intermittent Fe supply. Following a period of Fe shortage, plants take up excessive amounts of iron. They concluded that Fe is involved in chloroplast formation via protein synthesis, directly or indirectly.

Sideris & Young (163) agree with the former authors (96) that Fe is involved primarily in the synthesis of chloroplastic protein. They also agree that, following Fe deficiency, the Fe content did not correlate with chloro-

XUM

phyll content. They stress that Fe and N chlorosis differ only in extent and not in quality, presumably since both deficiencies impair the formation of chloroplastic protein. Wallihan (192) also postulated that Fe chlorosis of citrus is caused by a simple deficiency of iron.

It may be pertinent to theories of chlorosis that Shemin *et al.* (161) and Wriston *et al.* (204) have recently demonstrated that porphyrin is formed from "active" succinate and glycine. A succinyl intermediate and glycine were found to give rise to pyrroles which, in turn, gave rise to protoporphyrin. DeKock (45) noted that Fe is translocated primarily in the phloem and since Cu is concentrated in the phloem, Cu could thus exert maximal interference. In this connection, Whatley *et al.* (199) have reported that both Fe and Cu are concentrated in chloroplasts. Brown *et al.* (26) stated that plants differ with regard to the effect of Cu on the absorption and utilization of iron.

Brown & Wadleigh (29) stated that the HCO_3^- ion induced a pronounced accumulation of monovalent cations in leaves, but that treatment and chlorosis were not correlated with any particular cation or with the K/Ca ratio in bean and beet plants; rather, chlorosis correlated with monovalent cations or the Na+K/Ca+Mg ratio. Miller & Thorne (132) made an interesting observation to the effect that the rate of O_2 consumption by excised roots from a group of plants susceptible to lime-induced chlorosis was markedly reduced by bicarbonate ions; those resistant to this type of chlorosis were only slightly affected by bicarbonate. The inhibition of respiration, by bicarbonate ion, was not regarded to be the result of a direct inhibition of the cytochrome system. DeKock (44) noted that Ca/K ratio was lower in chlorotic leaves than in normal, green leaves. Weinstein & Robbins (197) recently concluded that Fe deficiency associated with high Mn (and/or low Fe) may be attributable to a direct competition between Mn and Fe for a position in the heme nucleus of the iron-containing enzymes—a suggestion that has been advanced by others previously.

Brown *et al.* (27) observed that plants did not develop Fe chlorosis upon the additions of P alone—only if Cu was also supplied. They stated that the difference of chlorosis susceptibility in one variety of soybean and nonsusceptibility of Thatcher spring wheat might lie in the fact that the dominant metabolic system in soybean involved Fe, whereas in wheat Cu was involved. In some plants Cu and P together decreased the absorption and utilization of Fe (as in PI-54619-5-1 soybeans), whereas in Thatcher wheat or Hawkeye soybeans, the plants showed no response to Cu and did not develop Fe chlorosis. According to Forster (66) plants that received the Cu salt of EDTA usually contained high concentrations of iron.

DeKock & Strmecki (41) and DeKock (45) noted that the ratio of P to Fe in healthy leaf cells varies within narrow limits, chlorotic leaves having either high (Fe deficiency) or low (Fe toxicity) ratios. He concluded that "the relative amounts of Fe and P absorbed by the plant determine whether

the plant will show chlorotic symptoms or appear healthy." Later DeKock (42) suggested that it is the level of soluble Fe in the cells of the root that determines how much Fe will be translocated to the leaf. With regard to the significance of Ca/K ratios in "lime-induced chlorosis," DeKock (43) observed that the Fe/P ratio of such plant material often correlated as well with the chlorosis as did the Ca/K ratio. He, therefore, did not regard this type of chlorosis as being a different type of chlorosis. The ratios of P to Fe and of Ca to K of chlorotic leaves of variegated plants were found to show the same trends from those of normal leaves as was shown by other forms of chlorosis [DeKock & Hall (44)].

Rediske & Biddulph (152) have also attached significance to the Fe/P ratio in chlorosis and have spoken therefore of "phosphorus-induced chlorosis." Working with plants in nutrient solutions, they concluded, on the basis of the following data, that both pH and P concentration play roles in the availability or unavailability of Fe in the plant: (*a*) entirely chlorotic leaves: 0.001 *M* phosphorus, 1.0 p.p.m. Fe as $FeCl_3$, pH 7; (*b*) veins only green: 0.0001 *M* phosphorus, 1.0 p.p.m. Fe, pH 7; (*c*) leaves all green: 0.0001 *M* phosphorus, 1.0 p.p.m. Fe, pH 4.

Crooke *et al.* (38) demonstrated that the Ni/Fe ratio was of importance in connection with chlorosis and, later, necrosis of leaf tissue. They stated that there was an indication of an Fe/P interrelationship under certain conditions and that high P may cause a reduction in internal availability of iron. They concluded, however, that the P status was related to the level of Ni in the plant.

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NITROGEN METABOLISM IN PLANTS: TEN YEARS IN RETROSPECT[1,2]

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Ten years ago Steward & Street (1) reviewed the nitrogen metabolism of plants. In 1947 the applications of chromatography had still to be made: this was the subject of a footnote. In 1950 Steward & Thompson (2) reviewed the initial contributions from paper chromatography and recognized the potentiality of this technique. By 1957 further outstanding advances in this field have been made by chromatographic methods. A large number of major plant constituents have been so discovered, and others continue to be recognized but remain to be identified. The significance of this newer knowledge should now be assessed.

Recognition of the role of keto acids in nitrogen metabolism can be seen in the writings of Chibnall (3), Vickery (4), and Virtanen (4a). The later implications of transamination were a major feature of the 1947 review. Again, however, the position has changed with the chromatographic recognition of a much wider range of acidic keto compounds than pyruvic, oxaloacetic, and α-ketoglutaric acids [Towers *et al.* (5); Virtanen & Alfthan (6, 7)]. Newly recognized keto analogues of amino acids, such as glyoxylic acid corresponding to glycine, succinic semialdehyde as possibly corresponding to γ-aminobutyric acid (8), pyruvic aldol corresponding to γ-hydroxy-γ-methylglutamic acid (9), and α-keto, γ-methyleneglutaric acid corresponding to γ-methyleneglutamic acid (10, 11), suggest that each amino compound in plants may be associated with its keto analogue.

Nitrate and ammonia have long been the inorganic forms of nitrogen most supplied to plants, with various other products of nitrogen or nitrate reduction regarded as conceivable intermediates. Urea, one of the first

[1] This review was completed in October, 1956.

[2] The following abbreviations will be used: ADP, adenosinediphosphate; AMP, adenosinemonophosphate; ATP, adenosinetriphosphate; DNA, deoxyribonucleic acid; DPN, diphosphopyridine nucleotide; RNA, ribonucleic acid; TMV, tobacco mosaic virus; TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide (reduced form).

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XUM

recognized natural nitrogen compounds, has never been regarded as a ubiquitous plant metabolite. The utilization of urea by ruminants and the rapidly expanding direct application of urea as a fertilizer, prompts a re-evaluation of the role of this compound and the manner of its assimilation.

Speculating upon the origin of life, it has been presupposed that primitive organisms were of necessity nitrogen autotrophic. This idea may now need revision since, under primitive geological conditions, amino acids could have arisen from methane, nitrate, and hydrogen by the action of ultraviolet radiation [Miller (12); Calvin (13); Urey (14)]. Fox (15) speculates upon the possibility that amino acids condensed, to form polypeptides, at elevated temperatures in a "pre-enzyme world."

A feature of the past decade has been the emphasis upon the enzymes involved in nitrogen metabolism, particularly in nitrate reduction, and group transfer reactions, and the mechanism by which energy is transferred to nitrogen compounds through phosphorylation. These aspects recently received attention by Webster (16) and were discussed in the two symposia held at the McCollum-Pratt Institute (17, 18). The trend has been toward the use of purified enzymes, with stress on the role of coenzymes, and also toward the investigation of mitochondria as inclusions involved in nitrogen metabolism. Helpful as these subcellular investigations are, the problems of nitrogen metabolism still concern cells and organisms as a whole, and they need to be related to growth and development on the one hand and to the various physiological functions on the other. Such correlations as were attempted in the earlier reviews (1, 2) will now be re-evaluated.

Certain major reference sources may be noted. In this series of reviews the articles by Wood (19), Wildman & Jagendorf (20), Webster (16), and Fogg (21) collectively bridge the period in question. Other reviews may be cited also (22 to 25). Although no authorative textbook account of nitrogen metabolism has been written since Chibnall (3), the earlier work of Prianishnikov was reprinted as late as 1951 (26). Certain major works deal with aspects of nitrogen metabolism in plants, at least in part. Plant proteins and nitrogen metabolism are discussed by Steward & Thompson (24) and Brohult & Sandegren (27). Certain general works on protein and amino acid metabolism deal with similar problems in animal systems and in bacteria [Gale (28); Fisher (29)].

A comprehensive study of biosynthesis in *Escherichia coli* by Roberts *et al.* (30) deals prominently with synthetic pathways, many of which involve nitrogenous compounds, in this and other microorganisms. In honor of Professor A. I. Virtanen a collective volume entitled *The Biochemistry of Nitrogen* appeared in 1955 (31), and many of the articles therein relate to metabolism of higher plants and bacteria.

Chromatographic and Analytical Procedures

A full discussion of present analytical methods based on chromatography cannot be attempted here. Several works, varying in completeness, describing chromatography are now available [Lederer & Lederer (32); Block,

Durrum & Zweig (33); Balston & Talbot (34); Cramer (35)]. It is desirable, however, to stress certain essential points, many of which were referred to by Steward, Zacharius & Pollard (25).

Qualitative procedures.—Since Dent, Stepka & Steward obtained the first two-directional chromatograms of plant nitrogen compounds (36), it has become increasingly evident that standardization of the chromatographic conditions is essential both in a given laboratory and, if possible, between laboratories, so that the findings of different workers may be compared. Certain features of the technique should especially be standardized: (*a*) the choice and purity of the solvents; (*b*) the water content of the solvents during the chromatography, especially as this is determined by temperature; (*c*) the length of travel of the solvent front, the effect of which tends to be eliminated by adopting alanine as an internal standard; and (*d*) precise equilibration of the paper and the solvents in the cabinet before the chromatography begins [Thompson *et al.* (37)]. In early chromatographic studies the paper was a chief variable, so that careful washing with acid, base, and water was necessary for almost all work. However, the Whatman chromatographic grades of paper now available may be used for many purposes without prior washing. Using standardized procedures, such as those stipulated (25), a wide range of nitrogen compounds, which react on paper with ninhydrin or which are detectable by other means [reaction with isatin, Erlich's reagent or other specific reagents, fluorescence, the starch-chlor-iodide method of Rydon & Smith (38)] can be detected chromatographically in alchoholic plant extracts. Granted such standardization of the chromatography, the results obtained on a variety of plants may be conveniently recorded in tables showing Rf values, or more precisely, R-alanine values; and these in turn may be illustrated by maps locating positions on chromatograms [Dent (39); Harris & Tatchell (40); Miettinen (41); Steward *et al.* (25)].

The need for precise standardization is evident from the following events. Inadvertently, when the tables and maps of Steward, Zacharius & Pollard (25) were compiled, the positions of β-alanine were recorded from observations on unidirectional chromatograms. However, when butanol-acetic acid follows phenol, the R-alanine for β-alanine is changed. Similarly the position for glutamic acid as recorded was somewhat in error, because inadvertently it related to earlier observations made using conditions in which the water content of the butanol-acetic acid was slightly different, the recorded position being in fact somewhat too low.

The utility of any solvent combination increases with its continued use. The two solvent combinations which best supplement each other, and with which most work has been done, use phenol-water in the first direction with collidine-lutidine-water in the second and, alternatively, phenol-water in the first direction with butanol-acetic acid-water in the second. Using the first of these solvent pairs, it is imperative to obtain the collidine and lutidine in a pure form. This presents more problems than the use of pure butanol-acetic acid.

Using their standardized procedure, Steward, Zacharius & Pollard (25)

XUM

recorded the chromatographic positions of some 85 known substances, many of which were naturally occurring. They also recorded some 84 naturally occurring but unidentified substances. Additions are continually being made to the latter category as new plants and new families are examined. For example, Fowden & Steward (42) noted 53 unidentified substances in the *Liliaceae* as well as eight newly recognized nitrogen compounds. Similar work with other plants in the laboratory of Professor Virtanen has been summarized (43). Other work of a similar nature has been carried out by Harris & Tatchell (40) for hops, and surveys have been made for the marine algae by Coulson (44) and Smith & Young (45).

Applications of chromatography to the quantitative analysis of plant extracts are of the following kinds: (*a*) the familiar procedure of Stein & Moore (46) originally separated the principal amino acids on starch columns; (*b*) this method was modified by substituting first Dowex 50×8 (47), and later Dowex 50×4 (48) cation exchange resins for the starch, displacing the amino acids in sequence from the resins with suitable buffers; and (*c*) various procedures have been recommended for the determination of nitrogen compounds after chromatography on paper.

Recognizing that many still unidentified nitrogen compounds occur in plants and the frequency with which quite different substances superimpose on paper chromatograms [Steward, Zacharius & Pollard (25)] and on ion-exchange columns [Moore & Stein (48)], any procedure which does not utilize the flexibility and resolving power of a two-directional system is limited in its application. Hence the techniques of Stein & Moore reach their full use in the examination of protein hydrolysates, the composition of which is rather definitely known, but their application to the substances found free in plants is more restricted.

Quantitative procedures.—Moore & Stein (48) recognized that asparagine and glutamine were not separated under their procedure and that glutamine was actually decomposed. These facts alone discourage sole reliance on these procedures for the analysis of the soluble fraction in plants. Bollard (50) and Boynton, Margolis & Gross (51) found that glutamine occurs in the soluble nitrogen fraction of the apple; Oland & Yemm (52) analyzed this fraction by the 1951 Moore & Stein procedure (47) which uses Dowex 50×8 and make no comment about the presence of glutamine. There is every reason to believe, therefore, that the glutamine of the apple was mainly converted by the procedure to pyrrolidone carboxylic acid, and any residue was determined as asparagine. Since substituted glutamines, now known to occur in plants (see pp. 81 ff. of this review), would also be lost under this procedure, it should only be used if such unstable compounds do not occur in the extracts.

The flexibility of the two directional paper procedures is, however, of no avail if it is applied with uncritical identification of substances by position alone. Wedding & Sinclair (53) claimed to find γ-aminobutyric acid in the hydrolysate of the protein of the Valencia orange and ornithine in the hydrol-

ysate of the Navel orange from work done on phenol-collidine chromatograms. Strikingly enough neither protein was reported to contain arginine. With this chromatographic system arginine may give either of two spots, depending on the pH, and these authors may have confused one of these with γ-aminobutyric acid and the other with ornithine. It is straining credibility too far to credit the protein of one orange variety with 10.6 per cent of its nitrogen as γ-aminobutyric acid and the other variety with 14.0 per cent of its protein nitrogen as ornithine, neither amino acid having been found previously to be a protein constituent. Further, arginine, which is universally recognized as a protein constitutent, was not reported in the protein fraction of either variety. Far-reaching claims of this sort should only be made if further corroborating evidence is given.

After ion-exchange separation, Block (54) applies both one- and two-directional paper chromatography for quantitative amino acid analysis, but this is probably no better than either method alone as applied by Hirs, Stein & Moore (49) or Thompson & Steward (37).

For the type and amount of solution to which it is applicable, the elegant quantitative procedure of Moore and Stein needs no further comment. Yemm & Cocking (55), however, make minor modifications to an earlier procedure of Moore & Stein (56), largely in the mode of application of ninhydrin, and they show calibration curves but no data from the application of their method to protein hydrolysates.

Different laboratories render the paper chromatographic technique quantitative for amino acids in different ways. Essentially, however, these fall into two groups. First, those methods in which the amino acids are determined quantitatively and reproduceably on the paper itself, after removing the chromatographic solvents; and secondly, the amino acids may be located by partial reaction with ninhydrin, prior to their elution from the paper for complete reaction in the test tube.

The procedure in this laboratory is still essentially that described earlier (37) whereby the amino acids are determined on the paper with a large excess of ninhydrin, by heating to 60° C. in an anaerobic (CO_2) atmosphere critically saturated with ethanol. In utilizing this procedure calibration curves for the different amino acids are necessary for the different solvent combinations adopted; that is, phenol-collidine-lutidine or phenol-butanol-acetic acid water, and also for the different chromatographic papers, i.e., Whatman Nos. 1 and 3.

Experience shows that a feature to be stressed particularly is the critical saturation of the chromatogram with alcohol vapor during the period when it reacts with ninhydrin (37). Furthermore, it is now known that the choice of solvent combinations has subtle effects on the color development with ninhydrin that were not at first fully appreciated. Some amino acids, e.g., histidine, are so much affected in their reaction with ninhydrin when collidine is used as the second solvent or in the ninhydrin spray, that their determination is difficult or impossible; but these substances can be determined using

XUM

the phenol-butanol system. On the other hand, some amino acids, e.g., aspartic acid, give more reproduceable calibration curves by the use of the phenol-collidine system than by the phenol-butanol system. The basic amino acids present the problem that their positions on chromatograms are somewhat affected by the conditions, particularly in acid hydrolysates as of protein. Experience, therefore, is necessary to interpret such chromatograms correctly. The efficacy of these procedures is such that they can be applied to the full examination of nitrogen compounds on very small amounts of material, as, for example, in the dissected apical growing regions of *Lupinus*, *Syringa*, and *Adiantum* [Steward *et al.* (57)]. They are also capable, when carefully applied, of yielding quantitative results as in the analysis of small amounts of pure protein [Thompson & Steward (58)].

Prior detection of the amino acids on the paper with ninhydrin, followed by elution and reaction in the test tube has been most carefully elaborated in the procedure recently described by Connell, Dixon & Hanes (59). This method is complicated by the very long washing of the paper (up to fifty days in lithium hydroxide solution) recommended prior to the chromatography. After chromatography, traces of ammonia are removed from the paper by use of a borate buffer, the amino acids detected by partial reaction with ninhydrin and, after elution, they are reacted in a test tube with a ninhydrin reagent which causes asparagine to react blue like other amino acids.

Chromatographic and other procedures are now so developed that it no longer seems necessary to determine the known amino acids by bioassay procedures.

The Soluble Nitrogen and Related Compounds in Plants: Their Occurrence and Identification

Amino acids.—Amino acids known to occur free and in proteins (or the acid hydrolysable insoluble fraction) are listed in Table I along with their naturally-occurring derivatives. The naturally-occurring and identified amino acids which occur in the free state, but which are not known to occur in protein, are listed in Table II with some of their naturally-occurring derivatives. These tables largely summarize information from the review of Steward, Zacharius & Pollard (25), the bibliography to which cites much of the supporting evidence. A reference citation in Tables I and II indicates that the substance in question has been identified since that review.

Only three of the substances mentioned in Tables I and II occur as their amides, i.e., aspartic acid, glutamic acid, and γ-methyleneglutamic acid. References to the occurrence of the amino acids in the form of their betaines can be found in the work by Guggenheim (73). Two of the amino acids listed are known to occur as phosphate esters; these are serine as a component of phosphatides, and aspartic acid [Black & Wright (74)], though as labile intermediates many or all amino acids may occur in this form. Hansen & Hageman (75) reported aspartyl and glutamyl derivatives of adenosinediphos-

TABLE I

AMINO ACIDS AND SOME OF THEIR NATURALLY-OCCURRING DERIVATIVES REPORTED TO OCCUR IN PROTEINS OR ACID-HYDROLYSABLE INSOLUBLE NITROGEN FRACTIONS*

Acidic Amino Acids:
- Aspartic Acid A, D, F
- Glutamic Acid A, D, F
- α-Aminoadipic Acid F
- γ-Hydroxyglutamic Acid F(60, 61)†

Basic Amino Acids:
- Lysine D, F
- Arginine D, F
- Histidine B, D, F

Cyclic Imino Acids:
- Proline B, D, F, N
- Hydroxyproline B, F, N

Aromatic Amino Acids:
- Phenylalanine D, F
- Tryptophan B, D, F, N
- Tyrosine D, F, N
- Monoiodotyrosine F
- Diiodotyrosine F

Sulphur Amino Acids:
- Cysteine D, F, S (62)†
- Methionine F, S

Hydroxy Amino Acids:
- Serine D, F
- Threonine D, F

Neutral Amino Acids:
- Glycine B, D, F, N
- Alanine D, F
- Valine D, F, N
- Leucine D, F, N
- Isoleucine F, N
- Sarcosine B, D, F, N
- Diaminopimelic Acid D, F

* *Key:* The protein amino acid in question also occurs as:

A = its amide	F = free amino acid
B = its betaine(s)	N = N-methyl derivative
D = its decarboxylation product(s)	S = S-methyl derivative

† Designates references since Steward, Zacharius & Pollard (25).

phate (ADP), and DeMoss *et al.* (76) reported activated compounds of amino acids with AMP.

The speed of progress may be appreciated by recognizing that in 1947 less then half of the substances listed in Table II were known to occur in plants. Moreover, many of the newly reported substances occur in nature in more than trace amounts or are widely distributed, as shown by the following examples. Pipecolic acid was isolated in the amount of approximately 14 gm. from 150 pounds of beans [Grobbelaar *et al.* (77)] and it occurs in most legumes and many other plants. γ-Amino butyric acid was isolated in the amount of approximately 6 gm. from 13 kg. of potato tuber and is ubiquitous in its occurrence [Thompson *et al.* (78)]. γ-Hydroxy-γ-methylglutamic acid represents approximately 90 per cent of the total soluble nitrogen of *Adiantum* apex [Steward *et al.* (79)], and also occurs in other ferns and in Liliaceae (42). Thus some plant or organ can be found in which almost any of the substances listed occurs in predominating amounts! This is very apt to happen in seeds or storage organs.

Keto acids in relation to amino acids.—Until recently the free keto acids of plants were presumed to be α-ketoglutaric, pyruvic, and oxaloacetic acids,

XUM

TABLE II

AMINO ACIDS OF PLANTS AND SOME OF THEIR NATURALLY-OCCURRING DERIVATIVES WHICH ARE NOT KNOWN TO OCCUR IN PROTEIN*

Acidic Amino Acids:
- γ-methyleneglutamic acid, A, D, F
- γ-methyglutamic acid, F (42)†
- γ-hydroxy, γ-methylglutamic acid, F (9)†
- α-aminopimelic acid, F (63)†
- γ-hydroxy, α-aminopimelic acid, F (64)†

Basic Amino Acids:
- α,β-diaminopropionic acid, P
- α,γ-diaminobutyric acid, F, P
- Ornithine, D, F
- β,ε-diaminocaproic acid, P
- δ-hydroxylysine
- Canavanine, F
- Roseoline, P (65)†

Sulphur containing amino acids:
- Cysteine, F
- Homocysteine, F
- Djenkolic acid, F
- Cystathionine, F
- Lanthionine, P
- S-methylmethionine, F
- S-methylcysteine, F (62)†
- S. methylcysteine sulfoxide, F (68, 68a)†
- Alliin, F (69)†

Cyclic Imino Acids:
- Methylproline, F
- Hydroxy, methylproline, F
- Pipecolic acid, D, F
- 4-hydroxypipecolic acid, F
- 5-hydroxypipecolic acid, F
- Baikiain, F
- Guvacine, F
- Azetidine-2-carboxylic acid F (66, 67)†
- Mimosine = Leucanol, F

Aromatic Compounds:
- Hydroxytryptophan, D, P
- Dihydroxyphenylalanine, F
- Phenylglycine, F

Hydroxy-Amino Acids:
- α-methylserine, P (70)†
- Homoserine, F
- β-hydroxy, α,α′-diaminopimelic acid, P
- Pantonine

Neutral Amino Acids:
- γ-aminobutyric acid, F
- Citrulline, F
- β-alanine, F
- Azaserine, F (71)†
- γ-hydroxyvaline, F (72)†
- 1-aminocyclopropane-1-carboxylic acid (72a)†

* *Key:* The substance occurs in the form of its:
A = Amide
D = Decarboxylation Product
F = Free Amino Acid
P = Antibiotic or Toxic Peptide
† Designates references since Steward, Zacharius & Pollard (25).

with the sporadic occurrence of glyoxylic acid. These conclusions were largely based on enzymatic methods and were influenced by knowledge of the Krebs cycle and its role in the metabolism of animals. This too-restricted view of the keto acids of plants was attributable to the limitations of the methods for their detection. By the prior conversion of keto acids to amino acids (5, 6, 7), which may be recognized chromatographically, it is now clear that other keto acids occur in plants. Some of these substances correspond to known amino acids, others give rise to amino compounds which still remain to be identified. Therefore, more acidic carbonyl compounds occur in plants than those required by the Krebs cycle or by deamination of known amino acids.

Two recent tabulations are of interest. First, Meister & Abendschein (80) synthesized a wide range of keto acids and then tested their conversion to amino acids through an analytical procedure which was essentially that used by Towers *et al.* (5). A compilation made in this laboratory and recorded by Rabson (81), lists the amino acid analogues for all the keto acids for which there is some evidence of natural occurrence. This is reproduced as Table III.

In addition to the identifiable naturally-occurring keto acids, a number of other acidic carbonyl compounds occur. Through their hydrazones and subsequent hydrogenolysis these give rise to still unidentified amino compounds. Towers, Thompson & Steward (5) noted some nine unidentified amino acid analogues of plant keto acids, the first one of which proved to be an artifact (109). Towers (110) and Rabson (81) observed many more.

Keto acids have been detected by their hydrazones [Cavallini *et al.* (111)]. The similarity in the chromatographic behavior of the hydrazones, with the complication that one such substance may give rise to two, or even three spots on a chromatogram [Isherwood & Jones (112)], limits the use of the hydrazones in the identification of keto compounds. Hence, the chemical conversion of the keto acid, through the hydrazone or the oxime, to a ninhydrin reactive substance is of special value.

General Implications of the Newer Amino and Keto Acids in Plants

It is necessary to assess the significance of the array of nitrogenous and related compounds now being discovered in plants. How are these newly discovered substances related to ideas of metabolic pathways? What is their significance in the nutrition of plants and animals? What, if any, significance do they have in such fields as taxonomy, morphogenesis, genetics, and plant pathology?

Human and animal nutrition.—The occurrence of unexpected nitrogen compounds in common food plants raises the question of their role in human and animal nutrition. Obviously these substances may, after absorption by the animal organism, be metabolized and used beneficially, be metabolized and prove injurious, or they may be merely excreted without change. Examples in all these three categories are known, as indicated below, but much further work is necessary. A recently discovered amino acid from plants which is used is γ-hydroxyglutamic acid; this rapidly gives rise to carbon dioxide in the rat, and to a variety of other substances derived from the residual carbon framework, notably aspartic and glutamic acids [Benoiton & Bouthillier (113)]. The possibility that the recently discovered S-methyl methionine, found in *Brassica* leaves and other plants, may have nutritionally beneficial effects has been noted [McRorie *et al.* (114)]. Nutritionally injurious effects attributable to the newly discovered natural nitrogen compounds are more conspicuous. Such examples are: leucenol in *Leucena glauca* [Yoshida (115)]; the *Lathyrus* factor [Schilling & Strong (116)]; and β-nitropropionic acid in *Indigofera*, [Morris *et al.* (117)], etc. The large array of

TABLE III

THE KETO ACIDS WHICH MAY OCCUR IN PLANTS, THEIR AMINO ACID ANALOGUES, AND THEIR METABOLIC ROLES*

Keto Acid	Amino Acid	Metabolic Role†	References‡
Glyoxylic	Glycine	Labelled early with C^{14} in photosynthesis	82
Pyruvic	Alanine	Links glycolysis with Krebs' cycle, etc.	
Mesoxalic	Aminomalonic	Possible intermediate in organic acid me' bolism	83, 84
Hydroxypyruvic	Serine	Linked to hexosemonophosphate pathway	6, 85
Oxaloacetic	Aspartic	Krebs' cycle intermediate	86
Acetoacetic	β-Aminobutyric	Intermediate in isoprene biosynthesis and fat metabolism	87
Succinic semialdehyde	γ-Aminobutyric	Glutamic acid and glutamine precursor	88, 8
α-Keto, β-hydroxybutyric	Threonine		7
α-Keto, γ-hydroxybutyric	Homoserine		7
Oxaloglycolic	Hydroxyaspartic	Possible intermediate in organic acid metabolism	89
Aspartic semialdehyde	α,γ-Diaminobutyric	Precursor of homoserine or azetidine-2-carboxylic acid	90
Diketosuccinic		Possible intermediate in organic acid metabolism	91
α-Acetolactic		Fermentation of pyruvic acid	92, 93
α-Ketoglutaric	Glutamic	Krebs' cycle intermediate and "port of entry" for N	
Glutamic semialdehyde	Ornithine		94
α-Ketoisovaleric	Valine		95
δ-Aminolevulinic		Intermediate in porphyrin biosynthesis	96
α-Amino, β-keto-adipic		Intermediate in porphyrin biosynthesis	96
γ-Methylene, α-ketoglutaric	γ-Methyleneglutamic		10, 11
γ-Methyl, α-ketoglutaric	γ-Methylglutamic		97
γ-Hydroxy, γ-methyl, α-ketoglutaric (Pyruvic aldol)	γ-Hydroxy-γ-methylglutamic	Inhibits Krebs' cycle	79
α-Keto, methylvaleric	Isoleucine		98
α-Ketoisocaproic	Leucine		99
α-Ketoadipic	α-Aminoadipic	Intermediate in lysine metabolism	6
Oxalosuccinic	α-Aminotricarbalyllic	Krebs' cycle intermediate	100
α-Keto, ε-aminocaproic	Lysine	Precursor of pipecolic acid	101
2-Ketogluconic,5-Ketogluconic		Glucose oxidation intermediates	102
2,5-Diketogluconic		Glucose oxidation intermediate	103
2-Keto,-3 deoxy, 6-phosphogluconic		Glucose oxidation intermediate	104, 105
α-Ketopimelic	α-Aminopimelic		6
α-Keto, γ-hydroxypimelic	γ-Hydroxyaminopimelic		6
2-Keto, 3-deoxy, 7-phospho, D-glucoheptonic		Intermediate in aromatic biosynthesis	106
Indolepyruvic	Tryptophan	Growth substance	107
Phenylpyruvic	Phenylalanine		
Hydroxyphenylpyruvic	Tyrosine		
Prephenic		Intermediate in aromatic biosynthesis	108

* Meister & Abendschein have listed numerous other keto acids prepared from naturally-occurring amino acids (80).

† Where not otherwise stated the keto acid has been regarded as related to its amino acid by transamination or deamination

‡ Keto acids and their analogues for which no authority is cited are generally-accepted constituents of plants.

ninhydrin reactive compounds in human urine, as observed by Westall (118) might imply that many of these plant constituents, when ingested, may be excreted. In fact, substances have been first recognized to occur in urine and then the natural occurrence in the food material has been detected. This occurred in the case of 5-hydroxypipecolic acid, which was seen as an unidentified substance on chromatograms of human urine, and later by association, its occurrence in the edible date was noted by Gartler & Dobzhansky (119) before the chemical identity was known (9). Ingested pipecolic acid may be excreted by the rat but is believed by Rothstein & Miller (120) to lie along the path from lysine to α-aminoadipic acid. However, the extent to which the relatively large amounts of organic nitrogen, represented by some of these newly detected plant constituents, are utilized by animals is still largely unknown.

Relations to mineral nutrition.—Since the use of chromatographic methods, some reference to this problem was made by Steward & Thompson (2). Effects of acute mineral deficiency on the mint plant were studied chromatographically by Crane (121). The most striking effect observed was caused by sulphur deficiency, which is associated in mint with a large increase in total soluble nitrogen and also with a preponderance in this fraction of arginine and glutamine. By contrast, free arginine was not detectable in mint plants grown under full nutrient conditions [Crane (121) cited by Steward & Pollard (8)]. Other examples of the accumulation of total soluble nitrogen and of arginine, due to sulfur deficiency, are also known, e.g., Mertz & Matsumoto (122). Extreme deficiencies of certain trace elements also resulted in changed composition of the soluble nitrogen fraction of tobacco plants, as described by Steinberg (123) from work done in this laboratory by Zacharius (124). A striking case of the accumulation, attributable to mineral deficiency, of a specific nitrogen compound not normally present is that of putrescine in potassium-deficient barley plants as investigated by Richards & Coleman (125). Putrescine would induce visible symptoms of potassium deficiency in plants with adequate potassium supply. Other plants require extreme deficiency before putrescine is detectable [Coleman & Richards (126)]. Holley & Cain (127) showed accumulation of arginine in iron-deficient and chlorotic blueberry, apple, and magnolia. Iron treatment restored green color to leaves and dissipated the arginine accumulation.

Effects of environmental factors.—From the early emphasis on carbohydrate-nitrogen relations in the determination of flowering, and the later recognition of the importance of photoperiod and thermoperiodicity in this respect, effects of these environmental factors on nitrogeneous metabolism of plants are to be anticipated. This has been studied in the mint plant, the development of which is strongly determined by the photoperiodic stimulus.

Crane (121) found that mint plants, grown under long- and short-day conditions in the greenhouse, showed quite different compositions of the leaves. The leaves of long-day plants had an abundance of glutamine and little asparagine, whereas the short-day plants contained less total amide,

XUM

especially asparagine, and a relatively larger amount of amino acid, particularly aspartic and glutamic acids. Later investigations by Rabson (81) have now indicated that the short- and long-day response is not as simple as first believed, because photoperiod also interacts with temperature, particularly night temperature. In fact, a low night temperature is very largely the determining factor in the prevalence of asparagine in the short-day plants. [See also Steward & Pollard (8).] In short, the photoperiod determines the proportion of total amide to free amino acid, and the ratio of glutamine and asparagine now seems to be a function of night temperature.

Rabson (81) has also observed such effects on mint plants after relatively short treatments, even periods as short as two and four days, and has also shown that these variables profoundly affect the keto acid content of the leaves, which changes in response to a single night at low temperature. The point of contact between keto acid and amino acid metabolism is, therefore, to be regarded as a focal point for the effects of environment on metabolism.

Relations to growth and development.—The earlier works of Nightingale on *Narcissus*, of Vickery on *Nicotiana* and *Narcissus*, and of Meiss on *Lupinus*, are well known (128, 129, 130) but these studies dealt with the few salient constituents that could then be determined. Now, however, the mobilization of reserve materials, as in seeds and storage organs, and their disappearance and re-utilization on germination or growth can be described by quantitative chromatography, and thus contribute to the knowledge of growth and development. Such studies have been made by McKee *et al.* (131), who studied the changes of nitrogen compounds in the development of the fruit of *Pisum;* by Fowden (132) with reference to γ-glutamyl compounds in *Arachis;* by Johnstone (133) with special reference to canavanine in *Canavallia;* by Miettinen (41) at different stages in the growth of the pea; and by Grobbelaar (134) with special reference to the effect of environment on the metabolism of different strains of pea.

In work by Hulme (135) a large part of the soluble nitrogen content of the unripe banana (var. Gros Michel from Honduras) fruit was found to be glutamine, asparagine, and histidine. During ripening the total soluble nitrogen of these fruits did not change, although free histidine increased and glutamine decreased. Many other such effects may be discovered by the methods now available.

Correlations between nitrogen metabolism and development should logically begin with the compounds in the growing point. For such a study Steward *et al.* (57) used the white lupin and *Adiantum* as a typical fern. Whereas the dicolytedonous plant contained, even in its growing points, most of the familiar nitrogen compounds, some however in small amount, the fern contained relatively few such compounds, some of which were present in conspicuous amount. One substance proved to be a new soluble nitrogen compound of plants, which Steward, Wetmore & Pollard (79) showed to be γ-hydroxy-γ-methylglutamic acid. This comprised as much as 90 per cent of the total soluble nitrogen.

As yet, ideas derived from the changes in the soluble nitrogen of the growing point concurrent with development are few. As far as the data go, they indicate that in the regions where growth by division is most prevalent the role of glutamine and glutamic acid over aspartic acid and asparagine is emphasized. They also tend to emphasize the role of the basic amino acids in the nitrogen metabolism of the dividing cells. In fact, these features were extremely localized even within the shoot apex of *Adiantum*. In this plant the relatively large, highly vacuolated outermost layer of the shoot apex had all the biochemical characteristics of the older nondividing cells, rather than those of the actively dividing relatively nonvacuolated tissue which occurred just beneath [Steward *et al.* (79)]. Such studies open the way toward investigations combining the use of chromatographic procedures to study nitrogen metabolism and histological procedures to relate the results to specific cells and tissues. When applied to the problems of development and morphogenesis, as they are affected by the environment, these methods should contribute much to knowledge of the metabolic basis for the behavior of growing regions.

Carrying the problem back even further requires consideration of the nitrogen metabolism of randomly dividing, proliferating, cells in contrast to their resting counterparts. Biochemical interpretations of dividing cells have been preoccupied with DNA and RNA, respiration and oxidation-reduction, —SH groups and enzymes [Stern (136)], but not with the detailed biochemical characteristics of the cells. Using tissue cultures, it has been shown that proliferating cells are different in their soluble and insoluble nitrogenous constituents. Commonly, mature nondividing cells, e.g., of the potato or the carrot, as they exist in the intact storage organ, are rich in free amino acids, especially asparagine and glutamine. When caused to grow by the use of growth factors found in coconut milk two changes occur. First, the balance between soluble nitrogen and protein is changed in favor of protein. Secondly, the balance between the amides also changes in favor of glutamine rather than asparagine, which disappears [Steward & Pollard (8)].

Similar differences between normal and cultured tissues have also been observed. Lioret (137) noted in various crown gall tissues an unidentified soluble nitrogenous constituent which was absent from the normal tissue. In Morel's laboratory (138) it was noted that winter-stored artichoke tubers were rich in arginine, but in the tissue cultured from these, arginine disappeared, protein increased, and seven unidentified, Sakaguchi positive substances appeared. One of these is now identified as γ-guanidobutyric acid by co-chromatography, and another is thought to be arginic acid. By use of C^{14}-labelled arginine, the conversion to proline and hydroxyproline was demonstrated [Dusanton (139)].

It is perhaps of even greater significance that the total protein of the growing cells has a different amino acid composition from the protein of the resting cells. Hydroxyproline occurs in the protein of the former in greater quantity than in that of the resting cells of the tuber or root (8, 24). Similar

XUM

effects have been observed in other proliferating cells (140). The full significance of this observation is not yet clear, although it has an interesting counterpart in the animal body, for collagen synthesis is stimulated in situations involving wound healing or tumorous activity, with a consequential stimulus to the metabolism of hydroxyproline (141).

The effect of floral initiation and development on nitrogenous constituents in the tulip bulb has been examined [Zacharius, Cathey & Steward (142)]. It had been noted (124) that arginine may account for up to 45 per cent of the soluble nitrogen in tulip. Floral initiation occurs during storage of the bulb. By frequent sampling, changes in the soluble nitrogen constituents of the bulb were determined at recognizable stages in the initiation and development of flowers. The soluble nitrogen in the bulb was remarkably unaffected during the period in which floral initiation occurred, and there was no sign that γ-methyleneglutamine or γ-methyleneglutamic acid were directly concerned in these events. However, when initiation was completed and floral development occurred, striking changes ensued so that the remaining constituents of the bulb shifted from an emphasis upon arginine (typical of the bulb) to an emphasis upon the soluble nitrogen compounds typical of the tulip leaf, particularly glutamine.

These selected examples emphasize the need for investigations which correlate the main facts of nitrogen metabolism with the effects of nutrition and environment, and to correlate them also with growth, differentiation, and development.

Taxonomic and genetic relationships.—Biochemical genetics might suggest that the occurrence of unusual compounds in specific situations may be subject to genetic control. The question then arises whether the newly recognized nitrogenous compounds have any taxonomic significance. Such problems require observations upon a wide range of plants and organs which inevitably disclose unidentified substances that can be recognized chromatographically and referred to only by letter or number, and an increasing array of substances that can now be identified.

Systematic surveys of this sort, however, are few. In the first selections of plants or organs for chromatographic study the taxonomic classification was rarely an important factor. First, the discovery in *Tulipa* of γ-methyleneglutamine and γ-methyleneglutamic acid (2, 143) and then of azetidine-2-carboxylic acid in *Convallaria* (66, 67) aroused interest in the Liliaceae and closely related plants. The recognition that γ-hydroxy-γ-methylglutamic acid may be the precursor of γ-methyleneglutamic acid and its amide (8) lent more interest to this study. Fowden & Steward (42) examined chromatographically many liliaceous plants and some from related families such as the Agavaceae. A detailed study was also made of the soluble nitrogen compounds that occur in the genus *Tulipa* (144).

γ-Methyleneglutamine was confined to a limited range of plants within the family, and even within the genus *Tulipa*, although the free acid was

more widely distributed. Azetidine 2-carboxylic acid was found in a number of plants besides *Convallaria*, but it was far from ubiquitous in the family. Such newly recognized substances as γ-hydroxy-γ-methylglutamic acid and γ-hydroxyglutamic acid were also noted. However, the only substance that occurred uniquely in all plants of this family and which could be detected chromatographically is still unidentified. [U-145 of Fowden & Steward (42)]. Fowden & Steward recognized that the accumulation of many of these unusual substances in particular plants or organs may be determined, not at the generic or specific level, but by mechanisms which require far fewer genes than are needed to determine the species, the genus, or the family. To this extent, therefore, these examples may be regarded as biochemical and genetic variants, the segregation of which may have been accentuated by a high degree of breeding and selection pursued for other purposes. Many of these substances, however, may also be normal metabolites in other plants in which they occur only below the level of chromatographic identification.

The presence in a fern, even in the apex of a fern, of a large amount of γ-hydroxy-γ-methylglutamic acid is strange enough; but the occurrence of a relatively small amount of the same substance in the tulip, accompanied by a relatively large amount of its dehydration product (γ-methyleneglutamic acid), does not admit of taxonomic explanation.

The existence of such compounds in diverse plants indicates that they form part of widespread and fundamental biochemical pathways. Even in the genus *Tulipa*, which is conspicuous for its content of the γ-glutamyl compounds, Fowden & Steward (144) concluded that these compounds may be superimposed upon an otherwise rather normal pattern of nitrogen metabolism, and that they may be regarded as somewhat remote from the metabolism most closely related to growth and development. This is substantiated somewhat by the difficulty that is being encountered in securing the entrance of C^{14} into the compounds in question from $C^{14}O_2$ and C^{14}-sugar [Wickson & Towers (97); Fowden & Steward (144)].

Studies (44, 45, 145) of plants from different levels in the vegetable kingdom present another facet of the problem; the earlier work of Allsopp (146) may be mentioned. Bollard has applied qualitative chromatographic procedures in a different way. Instead of analyzing tissue extracts he has applied the chromatographic method to the liquid content of the wood of trees, which is described as xylem or tracheal sap extracted from the woody tissues (147). Bollard has so investigated the nitrogen status of the apple tree (50); he has also examined plants from different families of angiosperms and gymnosperms (148). Broadly speaking, the compounds which Bollard has found in tracheal sap are far fewer than those which occur in alcoholic extracts of tissue. Most of the common amino acids were noted, and, in addition, the less obvious substances, citrullin, allantoin, and allantoic acid were prominent. However, the striking fact is that whereas Fowden & Steward (42) encountered as many as 53 unidentified substances in about

XUM

80 extracts of liliaceous plants, Bollard did not encounter many of the substances now known to occur frequently in plants: substances such an pipecolic acid, hydroxypipecolic acid, γ-methyleneglutamine, γ-methyleneglutamic acid, azetidine-2-carboxylic acid, γ-hydroxyglutamic acid, and various complex glutamyl compounds. And he did not recognize by letter or number any large number of unidentified substances such as the 95 or so found by Steward, Zacharius & Pollard (25). This raises an interesting problem.

If the content of tracheal sap represents the nutrients moving in this tissue for the nourishment of the shoot, then there is a degree of uniformity in these solutions as they occur in the woody plants which Bollard examined. However, γ-methyleneglutamine was actually discovered in the exudate from decapitated plants of the peanut (149), and Fowden (132) definitely regards this substance as important in the transfer of nitrogen from root to shoot. In those woody species of legumes, or those other plants in which the occurrence of such a γ-glutamyl compound might have seemed probable, Bollard did not encounter it in the tracheal sap; nor did he encounter any of the piperidine compounds which have recently become evident in the leaves, fruits, and seeds of legumes. This being so, secretion of nitrogen compounds into the tracheal sap may be selective.

It is difficult, therefore, to give simple explanations for the occurrence of these new nitrogen compounds and direct taxonomic relations are lacking. It can only be suggested that the biochemistry of the plants in question may have been established through evolution in ways which concern the operation of a relatively few genes. Therefore, in plant breeding programs, which change the properties of plants for other purposes (disease resistance, yield, etc.) consideration should also be given to the other biochemical effects which may occur concurrently.

In different investigations on nitrogen compounds preference may need to be given to the examination of different morphological regions such as leaf, fruit, seed, storage organs of root or shoot, tracheal or exuded saps, or even the growing points themselves. Frequently, however, fleshy storage organs, seeds, or fruits tend to accentuate a relatively few compounds, which occur in quantity, suggesting that incidental to the resting, or non-growing condition, substances accumulate at points in the metabolism which are temporarily blocked. In growing tissue passage through these substances may be so rapid that they do not accumulate. However, the occurrence of substances in large amount even in the plant growing point (as for example γ-hydroxy-γ-methylglutamic acid in the shoot of *Adiantum*) presents a dilemma, for it is hard to conceive of metabolic blocks in these growing regions which would immobilize as much as 90 per cent of the soluble nitrogen of the tissue!

Future investigation of the soluble nitrogen fraction of plants will, therefore, require emphasis upon genetic and taxonomic relationships as well as the developmental, nutritional, and environmental effects already mentioned.

Intermediary Nitrogen Metabolism: Implication of the Newly Discovered Glutamyl Compounds

This discussion will make reference only to certain topics in which rapid advances have been made. These topics are indicated by the subheadings which appear below.

γ-Methyleneglutamine as a third plant amide.—Ten years ago the consensus of opinion was that the amides of plants were confined to the two substances asparagine and glutamine which had figured so prominently in the history of nitrogen metabolism, although the occurrence of other amides, which had frequently been suggested, could not be entirely disregarded. Even urea, the most prominent amide in animals, was not generally regarded as a plant constituent and neither this substance nor the ureides were prominently considered in the intermediary metabolism of nitrogen in plants. As late as 1950 (2), in spite of the occurrence of a large number of still unidentified nitrogen compounds, the question could only be posed as follows: "Will previous suggestions that there are more plant amides than glutamine and asparagine be verified?" The existence of a third plant amide was, however, quickly established. In the earlier review of 1950 a substance had been described on paper chromatograms of the tulip bulb (unknown No. 12); this was unstable in acid solution and accounted for two substances on the chromatograms. They were later shown to be related as the free acid and its amide [Zacharius (124)]. From the exudate of decapitated peanut plants (*Arachis hypogea*) Done & Fowden (149) isolated a substance which they eventually identified as γ-methyleneglutamine. This identity was later confirmed by synthesis [Wailes, Whiting & Fowden (150)]. After the work of Done & Fowden, the substance which had been isolated in a pure state from the tulip (124) was quickly shown to be identical with the substance obtained from the peanut. Thus, two new plant constituents were identified and the existence of a third plant amide was finally established. Later evidence shows that these substances occur in other plants. Harris & Tatchell (40) found them in hops. Fowden & Steward (42) have indicated the frequency of the occurrence of γ-methyleneglutamine in liliaceous plants, for it occurred in 17 out of 88 plant extracts examined. However, most of these (15 in number) represented different species of *Tulipa*, and the two remaining examples of its occurrence were from the closely related genus *Erythronium*. Wickson & Towers (97) found γ-methyleneglutamine only in *Lilium regale* out of six *Lilium* species examined and Fowden & Steward (42) noted that although several other γ-substituted glutamyl compounds were conspicuous in *Lilium*, γ-methyleneglutamine was not detected in the three species examined.

Compounds related to γ-methyleneglutamine.—The first related compound to be considered was the decarboxylation product of γ-methyleneglutamic acid, namely γ-amino-α-methylenebutyric acid. Fowden & Done (151) compared a small amount of an isolate from peanut plants with enzymatically decarboxylated γ-methyleneglutamic acid. So far as this comparison went it

XUM

indicated the occurrence of γ-amino-α-methylenebutyric acid in the peanut plant. To the present it has not been reported in other plants. Hence it is much more restricted in occurrence than the parent substance γ-methyleneglutamic acid.

The next compound related to γ-methyleneglutamic acid to be considered was the corresponding keto acid, α-keto-γ-methyleneglutaric acid. Fowden & Done (152) observed that γ-methyleneglutamic acid would participate in transmination reactions *in vitro*, and therefore presumed that the keto acid analogue of the amino acid must exist. Using the technique of converting naturally-occurring keto acids to their amino acid analogues (5), Towers & Steward (10) detected a keto acid in the tulip which corresponded to γ-methylglutamic acid. However, the technique then adopted did not distinguish between the keto acid analogue of γ-methyleneglutamic acid and of γ-methylglutamic acid, since both hydrazones on hydrogenolysis would give the same amino compound. At the time the presumption was made that the natural keto acid was the analogue of the γ-methyleneglutamic acid. In the light of later evidence that γ-methylglutamic acid frequently occurs [Fowden & Steward (42); Virtanen & Berg (153)], this was not fully justifiable. From the peanut, Fowden & Webb (11) isolated a keto acid, in the form of its hydrazone, which was chemically identical with the hydrazone of a keto acid prepared enzymatically from γ-methyleneglutamic acid. From all this evidence and the now prevalent opinion that the keto acid analogues of amino acids quite frequently occur in the free state, the occurrence of α-keto-γ-methyleneglutaric acid can be presumed for the plants in which the corresponding amino acid is typical.

Later, another γ-substituted glutamic acid was recognized. This compound could, theoretically, be of even more general significance, because it may lead to many of the other γ-substituted glutamyl compounds which have been detected. This compound is γ-hydroxy, γ-methylglutamic acid. γ-Hydroxy, γ-methylglutamic acid was first recognized as an unidentified spot on paper chromatograms of the shoot apex of *Adiantum* [Steward *et al.* (57)]. It occupied a position close to serine on the chromatograms studied but, on later examination, proved to be the substance in question (9, 79). This amino acid was prepared, by a sequence of reactions described, from pyruvic aldol and, though the synthetic material was a mixture of stereoisomers, its chromatographic behavior and analysis were identical with those of the naturally-occurring product. Furthermore, substances which behaved chromatographically as γ-hydroxy, γ-methylglutamic acid have also appeared in many liliaceous plants (42). By the technique of converting a keto acid to its amino acid analogue, it was possible to establish that the pyruvic aldol also occurred in the free state in *Adiantum* (79).

γ-Hydroxyglutamic acid and related substances.—From a different line of investigations γ-hydroxyglutamic acid is now known to occur free in plants. This was established by Virtanen (61), using extracts from the cultivated phlox (*Phlox decussata*). The isolate was reduced with hydriodic acid to

glutamic acid, and the analysis of the isolate fitted the empirical formula of hydroxyglutamic acid. Oxidation to aspartic acid indicated the "γ" position of the hydroxyl group. γ-Hydroxyglutamic acid was chromatographically recognized in three liliaceous plants and was shown to be chromatographically identical with the substance as it occurs in *Phlox* and with a synthetic product which was obtained from Professor L. Bouthillier of the University of Montreal [cf. Benoiton & Bouthillier (154)].

The recognition in *Phlox paniculata* of a substance which on hydrolysis yields γ-hydroxyglutamic acid, equimolar quantities of ammonia, and a C_5 carbon residue [Pollard & Steward (155)] shows another related substance in plants; this appears to be a substitution product of a fourth amide, namely hydroxyglutamine. This substance, still incompletely identified, has only been isolated in the pure form from *Phlox*, but chromatographic evidence is consistent with its occurrence in *Hemerocallis* and in certain other liliaceous plants.

New glutamyl compounds and metabolic pathways.—Thus, at least nine γ-substituted glutamyl compounds, or their derivatives, have been discovered in recent years, and two of these appear to be amides. However, still other glutamine derivatives, other than those which are γ-substituted, are now coming to light. Evidence on this was summarized by Steward & Pollard (8), who emphasized that the number of natural substitution products related to glutamic acid now far exceeds those for aspartic acid and asparagine.

The newly discovered metabolites often seem unresponsive to physiological variables that drastically change metabolism. Attempts to label these substances with C^{14}, through photosynthesis or other means, have proved inefficient. At least four known attempts in three different laboratories to incorporate C^{14} into γ-methyleneglutamine or γ-methyleneglutamic acid have not yielded products of high activity. But, whenever these substances have been investigated enzymatically, they have been found to participate in reactions (transamination, oxidation, decarboxylation) consistent with their possible metabolic role. Present indications are that many of these substituted glutamyl compounds are somewhat remote from normal metabolic pathways and, in plants in which they appear, their occurrence may be superimposed upon more typical metabolism of amino acids and protein (144). It is also true that other substituted amides which are unrelated to amino acids, or their metabolism, are now being found in plants by Crombie (156), who has detected butylamides of unsaturated organic acids. Various antibiotics are also amides [cf. Bracken (157, p. 121–22)]. One may expect that amides of other organic acids may also occur, though there have not been visible means for their ready detection.

Metabolic pathways, known or conceivable, which flow from pyruvate via pyruvic aldol, via γ-hydroxy-γ-methylglutamic acid, emphasize the variety of substances which can be related to these possible precursors. This includes such newly discovered natural substances as the substituted pro-

XUM

lines and citramalic acid. These and other relationships are indicated in Figure 1 of Steward & Pollard (8).

Another substance intimately related to glutamic acid is the now familiar γ-aminobutyric acid, which was one of the first prominent substances to be identified by chromatography. First thought to be a decarboxylation product of glutamic acid, this substance now acquires a different significance. When supplied in the radioactive form to tissue cultures the carbon it contains appears readily in glutamine, whereas the conversion of labelled glutamine to γ-aminobutyric acid is slight [Steward, Bidwell & Yemm (158)]. Furthermore, nonphotosynthetic fiaxtion of $C^{14}O_2$ by potato tissue slices resulted in the appearance of C^{14}-labelled glutamic acid, suggesting the carboxylation of γ-aminobutyric acid (8). Since γ-aminobutyric acid did not arise primarily from decarboxylation of glutamic acid, its origin from succinic semialdehyde is suggested: this is consistent with the known transmination reactions, catalyzed by plant enzymes (88, 159), of γ-aminobutyric acid. This evidence suggests that, in some plants, γ-aminobutyric acid may lie along a path of synthesis en route to glutamic acid and glutamine rather than the reverse, the latter being the case in the metabolism of barley leaves [Naylor & Tolbert (160)].

This trend of thought attracts attention to pyruvic aldol as a pivotal metabolic substance. In purified enzyme systems pyruvic aldol is known to be an inhibitor of the Kreb's cycle enzymes, specifically of the α-ketoglutaric acid decarboxylation [Montgomery & Webb (161, 162)]. Thus, the presence of pyruvic aldol might modify the operation of the Kreb's cycle and divert metabolism into the areas discussed above.

Carbamyl and Guanidyl Compounds: Their Occurrence and Metabolism

Historically, ideas of intermediary nitrogen metabolism have centered around ammonia, amino acids, particularly the dicarboxylic amino acids, and their amides as the pivotal substances. Despite the early observations that urea, guanidine and their derivatives occur in plants, these substances have not commonly been closely integrated with the main paths of metabolism. Present trends indicate that they are of greater physiological significance than has been generally recognized.

Urea.—The early recognition of urea in animal metabolism and its ready isolation led naturally to consideration of its role in plants. Liebig probably missed, through his over-emphasis on the role of ammonia in plant nutrition, an early observation of urea and ureides in plants. This occurred as follows. He noted in 1834 the copious amounts of ammonia obtained by treating evaporated maple sap with lime. This he attributed to the release of ammonia from neutral salts (163). So anxious was Liebig to avoid any suspicion of contamination of the maple sap that "vessels which hung upon the trees in order to collect the juice were watched with greater attention on account of the suspicion that some evil-disposed persons had introduced urine into them,

but still a large quantitity of ammonia was again found in the form of neutral salts" (163, p. 32). It is now clear that this ammonia really came from allantoin and allantoic acid which occur in the maple sap [Mothes & Englebrecht (164) and Bollard (148)]. Through the classical period of Pfeffer, Schulze, and Prianischnikow references to urea are infrequent, this probably resulting from the accepted excretory role of urea in the animal which has no counterpart in plants. Despite this, however, observations of the occurrence of urea in plants were made, and by 1911 these were summarized by Verschaffelt (165).

As late as 1955 Tracey (166) recognized that this was a much neglected area and compiled a comprehensive table showing the widespread occurrence of urea and ureides and their enzymes in 29 families of angiosperms. (Incidentally, Tracey attributes claims of free urea in higher plants to decomposition of the allantoic acid.) Without repeating Tracey's more exhaustive citations, it is appropriate to refer to the main events in the history of urea and ureides in plants. By 1912 Fosse had shown the somewhat general occurrence of urea, using chemical tests and by isolation of dixanthyl urea (167). Fosse even grew maize aseptically and demonstrated urea in various parts of the organism (168). He considered urea to be an end product of purine metabolism and not as an initial source of nitrogen or a general metabolite (169). Between 1927 and 1932 Klein & Taubock, using urease to demonstrate free urea and ureides, found urea most conspicuous in the early stages of germination, in the etiolated plant, and in immature seeds (170). These authors recognized that this urea could originate from arginine rather than by oxidation of purines (171). Damodaran & Venkatesan (172) investigated urea formation in germinating seedlings of certain legumes, finding considerably more urea than could be accounted for by the breakdown of arginine. Reifer & Melville (173) believe that a urea precursor exists in grasses and this forms urea when the foliage is dried. These authors showed that urea furnished to the roots or leaves is rapidly converted to glutamine. Studies of the intimate metabolism of urea are now possible through the use of urea labelled with C^{14} and N^{15}.

Although anilides are known to have physiological properties (viz. acetanilide or "antifebrin"), such compounds seem not to have been isolated from plants nor has their activity been invoked. The finding, therefore, that carbanilide (diphenylurea) was not only obtained from a large batch of coconut milk, but also shown to have growth promoting properties for carrot, artichoke, and potato tissue cultures (174), and other systems (175), is both novel and interesting in view of the toxicity of substituted urea herbicides. The invariably positive tests for aromatic amines (176) after treatment with alkali gives some indication of the content of this class of substance that may be expected in plants.

Guanidine.—Some observations indicate that guanidine occurs free in plants. However, compounds of guanidine range from arginine itself, which is widespread in the free state and an important constituent of protein, to

XUM

such compounds as galegin (isoamyleneguanidine) which occurs in *Galega* (177), canavanine which is a guanidine derivative of homoserine and is present in jackbeans (*Canavallia ensiformis*) (178), soy beans (*Glycine hispida*) (179), and *Colutea arborescens* (180), and streptidine which is part of the streptomycin molecule and is a compound of inositol and guanidine (181). *Streptomyces* also produces γ-guanidobutyramide by an unusual oxidation of arginine [Thoai *et al.* (182)]. Using paper chromatographic methods for substituted guanidine derivatives, [as described by Roche *et al.* (183)], Mourgue *et al.* (184) have furnished evidence that guanido compounds other than arginine occur in castorbeans (*Ricinus*). These compounds are agmatine, the decarboxylation product of arginine, γ-guanidobutyric acid, and two unidentified substances. The occurrence of other guanido compounds in tissue cultures of Jerusalem artichoke has previously been noted (cf. p. 77). Since guanidine and some of its related compounds do occur free in plants, the extension of chromatographic methods to their detection should be profitable [cf. Makisumi (185)].

Techniques for the detection of carbamyl and guanidyl compounds.—The earliest methods involved the actual isolation of the compounds in question. Later methods involved the action of enzymes. Urease is commonly believed to be specific for urea and it is used to determine urea in the free state and bound urea released on acid hydrolysis. This specificity is now questioned [cf. Tracey (166, p. 125)]. While this test is very sensitive for free urea, it does not distinguish between different ureides.

Enzymic procedures for the guanidine compounds are less convenient. Arginine can be determined by the combined action of arginase and urease (186). A less specific enzyme, heteroarginase, will release urea, detectable by urease, from a range of guanidine compounds (187). An incompletely characterized arginase exists in plants (188).

Ureides and guanidine compounds may be separated on paper by the usual procedures of chromatography, but the reagents which are in use for their detection on paper are not entirely specific. These are Erlich's reagent (*p*-dimethylaminobenzaldehyde) for urea and ureides and Sakaguchi's reagent for substitute guanidines. Both classes of substance may be located on the papers by the nonspecific method of Rydon & Smith (38). The use of ninhydrin may be extended to include guanidine compounds by treatment with alkali after ninhydrin (25).

The urea cycle in plants.—All the intermediates of the classical urea-ornithine-arginine cycle have been found somewhere in plants, but the evidence for the cycle stems mainly from the techniques of biochemical genetics in relation to *Neurospora*, i.e., from the requirement for specific substances to overcome the effects of genetic blocks [Srb & Horowitz (189)]. More recently, however, Coleman & Hegarty (190) have shown that C^{14}-ornithine may be rapidly and generally metabolized in barley and C^{14}-citrulline labelled in the carbamyl group forms arginine and urea. Kasting & Delwiche (191) applied C^{14}-labelled compounds to higher plants (water-

melon) known to contain citrulline. They claimed some evidence for the entrance of the carbon from C^{14}-citrulline and C^{14}-arginine into the appropriate intermediates of the cycle. Curiously, however, they state that urea is not used directly but is hydrolysed, its carbon being dispersed into many compounds.

This type of evidence, however, still leaves the operation of a cyclical process to be demonstrated and its metabolic significance in higher plants is not obvious, since plants do not excrete urea. The donation of ammonia and CO_2 to ornithine to give citrulline was previously obscure. In the presence of enzymes, ornithine and carbamyl phosphate yield citrulline [Jones *et al.* (192)]. Carbamyl phosphate thus promises to be an intermediate because it can donate the carbamyl group and contains an energy-rich phosphate bond. However, the origin of carbamyl phosphate is still uncertain, but it appears to involve carbamyl glutamate as a link between the ammonia, the CO_2 and the source of energy (ATP) in liver [Ratner (193)]. For a discussion of these reactions in relation to mitochondria see Lindberg & Ernster (194, p. 85–88).

By the use of N^{15}-labelled glutamine, Ory, Hood & Lyman (195) showed that the amide group of glutamine furnished the nitrogen of the carbamyl group of citrulline (possibly via carbamyl phosphate). In *Chlorella* (196), as in kidney [Borsook & Dubnoff (197)], aspartic acid furnishes the nitrogen for the conversion of citrulline to arginine via arginino-succinic acid.

Suggestions of the occurrence of free ornithine, or even compounds of ornithine, are few (25, 198). The evidence does suggest that free citrulline occurs frequently and in quantity, even in plants other than the Cucurbitaceae in which it was first discovered by Wada (199). Virtanen (200) found citrulline in the root nodules of *Alnus* and isolated this material. Bollard confirmed this observation and extended it, on chromatographic evidence, to show that free citrulline is a frequent constituent of the tracheal sap of woody plants (148). Thus, the mounting evidence for the occurrence of the intermediates of the urea-ornithine cycle, suggests that this also operates in higher plants.

The direct utilization of urea.—The implications of the now widespread use of urea as a fertilizer are as follows.

The first assumption was that the use of urea was mediated by urease and that it served as an alternative source of ammonia, the carbon being lost as carbon dioxide. Hinsvark, Wittwer & Tukey (201), using C^{14}-labelled urea, gave direct support to this idea since in the dark, C^{14} originally furnished as urea passed into the carbon dioxide. Plants differ in the rate at which they decompose urea and, where the conversion to CO_2 and presumably to ammonia was rapid, injury seemed to be greater, the idea being that injury attributable to urea is really caused by ammonia.

The implication of Hinsvark, Wittwer & Tukey's work is that none of the carbon of urea accompanies the nitrogen. Whereas the previous workers did not detect fixation of the carbon of urea, Webster, Varner & Gansa

XUM

(202), working in "weak white light" with C^{14}-labelled urea, definitely detected incorporation of the C^{14} of urea into nitrogen compounds. They also compared the incorporation of the C^{14} from urea and from sodium bicarbonate in the presence of ammonia and stressed the similarity in the compounds which incorporate carbon from these two sources.

Urea furnished to apple leaves appears mainly as glutamine [Boynton, Margolis & Gross (51)]. This also occurs in aerated potato slices, although the utilization of the urea is determined, in part, by the origin of the potato tissue. Slices cut from some tubers freely convert urea to glutamine whereas slices cut from others tend to accumulate only urea [Steward & Pollard (8)]. From Webster's work (202) one cannot tell the extent to which the nitrogen of urea was utilized because the initial tissue was not analyzed. In Webster's work, regarding incorporation of C^{14} as a measure of the synthesis of compounds from urea would imply that other substances than glutamine, notably aspartic acid, asparagine, glutamic acid, and glycine, were also involved. Whereas Webster *et al.* stressed the similarity in the use of C^{14} from urea and bicarbonate, Steward & Pollard (8) stressed that there may be something quite unique about the incorporation of carbon from urea which distinguishes it from the incorporation of CO_2. In aerated potato discs urea led to a greater increment of glutamine than was obtained by the use of ammonium salts, and when C^{14}-labelled urea was furnished, most of its carbon appeared in glutamine. Reifer & Melville (173) also comment that "urea was probably involved in the metabolism of the plants without prior hydrolysis to ammonia and carbon dioxide," which is in accord with the above suggestion.

Freiberg & Payne (203) studied the uptake of C^{14}-urea in the banana plant. Surprisingly, there was no evidence for urease in the leaves, although it could be detected in the shoot growing point; this implies that urea is absorbed by the leaves, translocated a great distance to the shoot apex where it is acted upon by urease. Alternatively there must be some method of urea utilization in the banana independent of urease.

Walker, working with *Chlorella*, points out that this organism will grow on urea as the sole nitrogen source even though urease could not be detected (196). The incorporation of urea-carbon and -nitrogen into guanine by *Chlorella* and *Scenedesmus* has been demonstrated by Ellner & Steers (204). Apparently, however [Fox (15)], the thermal condensation at pH 9.0 of urea and malic acid to ureidosuccinic acid (carbamyl aspartic acid) has long been known [Lippich (205)] and this in turn by condensation and hydrogen transfer, gives orotic acid, which may well lead to pyrimidines.

Allantoin and allantoic acid.—Compounds of this nature, long known to occur in plants, were mainly regarded as oxidation products of purines and not directly related to protein or nitrogen metabolism. Reference to Tracey (166) may be made for the older work. Reconsideration of the role of these substances derives from the work of Mothes & Englebrecht, who stressed the occurrence of allantoin and allantoic acid in such plants as *Acer* (164), *Symphytum*, and other Boraginaceae (206), particularly in the tracheal and

root bleeding sap, and also in roots, stems, branches, opening leaf buds, and flower buds; also in the cotyledons of *Phaseolus* (207). In some circumstances allantoic acid can account for all of the ureide nitrogen and, according to Mothes, these compounds may assume the role in storage and translocation, which in many plants is performed by asparagine and glutamine. In the bleeding sap from *Acer* the total allantoin nitrogen accounted for a high percentage (70 to 100 per cent) of the total soluble nitrogen. The rapid changes with time which these compounds undergo in the plants investigated leads Mothes to conclude that they are more metabolically active than has been recognized (164).

The techniques of Mothes were chemical in nature. The determination of allantoin and allantoic acid by its nitrogenous hydrolytic products is subject to confusion with any sources of labile ammonia on hydrolysis, ranging from known amides to still unidentified compounds. Mothes, therefore, focused attention on the glyoxylic acid which arises on hydrolysis; this he determined by the red coloration that forms with alkaline phenylhydrazine. If the specificity of the chemical hydrolysis of the ureide was in doubt some use was also made of enzymatic methods. In view of these difficulties the precision obtainable by the use of chromatographic procedures becomes important. In later work from Mothes' laboratory, Reuter & Wolffgang (208) used chromatography to determine the nitrogenous components in the bleeding sap from *Betula;* this included the amino acids and also citrulline and allantoic acid, the latter determined by Ehrlich's reagent.

Bollard (148) has determined the chromatographic behavior of allantoin and allantoic acid more precisely, using synthetic allantoic acid and has shown that they may be satisfactorily detected on phenol-butanol-acetic acid chromatograms. The conclusions of Mothes *et al.* have been broadly confirmed by Bollard. The presence of the ureides in the tracheal sap was emphasized, but never to the exclusion of amino acids and amides. Since allantoin and allantoic acid occurred in trachial sap from 23 out of 103 species examined, representing 94 families of dicotyledons, Bollard regards them as probably important in translocation. Species and families tend to fall into two groups with reference to the composition of tracheal sap with citrulline or allantoin and allantoic acid tending to be conspicuous.

In the animal body allantoin and allantoic acid are excretory end products of purine metabolism, but in plants they seem to be more immediately concerned with synthesis and use of the nitrogen they contain. Recognizing that glyoxylic acid occurs free in plants and is related to glycolic acid, which is involved in photosynthesis (82), it can be seen that allantoin and allantoic acid might derive from glyoxylic acid, with urea as a possible nitrogen donor. This idea could be tested by the use of C^{14}-labelled compounds.

Arginine and other carbamyl or guanidyl compounds.—Arginine accumulates when protein synthesis is arrested, as for example in certain storage organs such as the tulip bulb (142), or as a result of certain mineral deficiencies, e.g., of sulphur (8, 121). Recently Holley & Cain (127), have shown the

XUM

accumulation of arginine resulting from deficiency of iron in apple, blueberry, and magnolia. Arginine also accumulates in the sclerotia of the fungus *Sclerotinia* [T. Sproston, confirmed by Pollard (209)] and in the gametophyte and the sporophyte of *Lycopodium cernuum* (209). Arginine is related to γ-guanidobutyric acid by oxidation (210) and it, in turn, by the action of heteroarginase (187), releases urea with the formation of γ-aminobutyric acid. However, the evidence for these reactions is only from animals.

Finally, and by analogy with recent evidence from animal systems, it is now possible that the guanidyl moiety may be passed intact from one compound to another. Thus, a variety of guanidine compounds might be interchangeable via some metabolic pool, in a fashion similar to the mobility of amino nitrogen via transamination (186).

Canavanine is structurally related to arginine by the replacement of the δ-methylene group by oxygen. This substance is best known from jackbean (*Canavallia ensiformis*); it is a competitive inhibitor of arginase and causes a canavanine inhibition of growth in *Neurospora* which is reversed by arginine (211). In *Canavallia* canavanine accumulates only during seed formation and is depleted during germination (133).

Thus, urea and the ureides and guanidine and guanidyl compounds comprise molecules which are rich in nitrogen, but because they have not been considered closely related to protein metabolism comparatively little is known about their behavior, at least in comparison with the amino acids and their amides.

Imino Acids: Their Occurrence and Biogenesis

To proline and hydroxyproline have been added methylproline [Hulme & Arthington (212)] and methylhydroxyproline as probable natural constituents [Hulme (213); Urbach (214)].

The relation of proline to glutamic acid and ornithine is well known (cf. 94) and by a similar series of reactions the natural origin of pipecolic acid from lysine is now well established in plants (101, 215). Other piperidine compounds occur as natural products: baikiain (tetrahydropicolinic acid or dehydropipecolic acid) [King *et al.* (216); Grobbelaar *et al.* (9)]; 5- and 4-hydroxypipecolic acid [Virtanen & Kari (217, 217a); Grobbelaar *et al.* (9)]; and the substance guvacine, which is a dehydronipecotic acid, has been known as a natural product for a longer period [Freudenberg (218)]. Picolinic acid, the fully dehydrogenated acid corresponding to pipecolic acid, occurs in a fungus *Piricularia oryzae* (219); thus the series—picolinic icid, baikiain, pipecolic acid—represents progressive stages of reduction of the pyridine ring. Conversely it might represent progressive stages in the formation of the pyridine from the piperidine ring. Piperidine itself occurs in plants (cf. 25) and may be responsible for toxicity to livestock (220).

Recently, the first example of a naturally-occurring azetidine carboxylic acid appeared; this could be related to α-γ-diaminobutyric acid (221, 222) and to aspartic acid or aspartic semialdehyde which is known to occur in

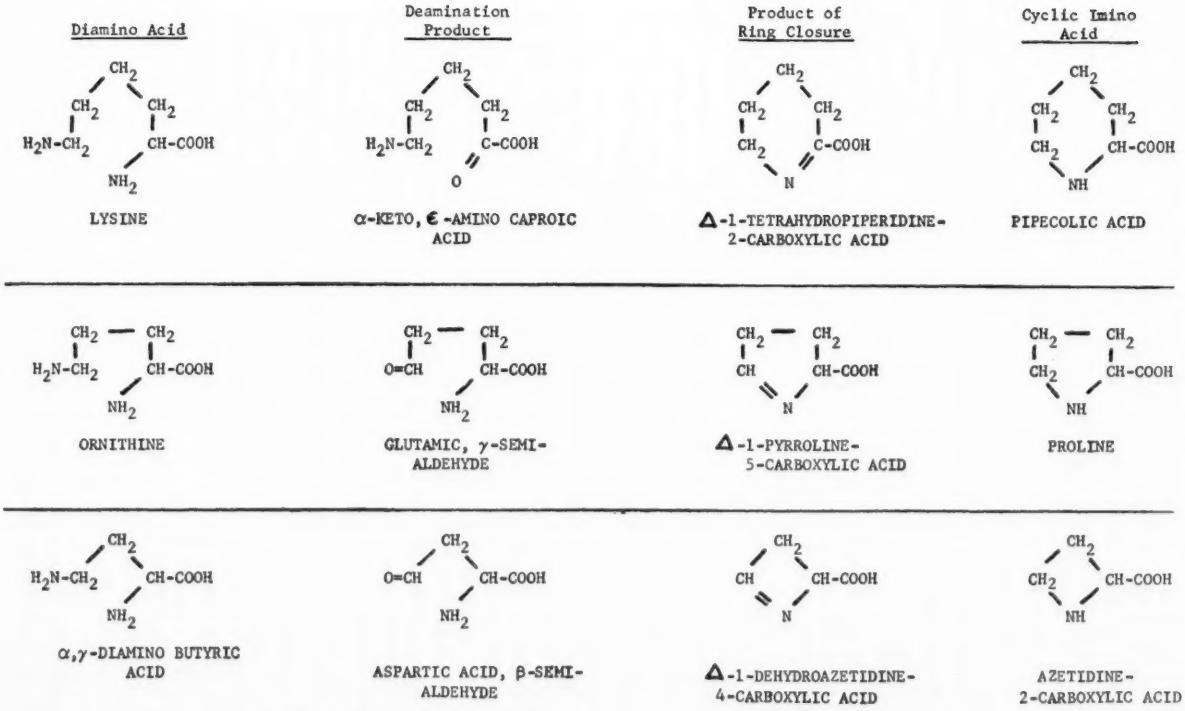

FIG. 1. Metabolic intermediates related to some naturally occuring cyclic amino acids.

yeast (90). The known and hypothetical relationships between these compounds are shown in Figure 1, in which the general pattern of the reactions is that of the conversion of ornithine to proline and glutamic acid. The publication (215) dealing with conversion of lysine to pipecolic acid in *Phaseolus* recognized, but did not identify, α-aminoadipic acid as a subsequent product; this has now been done [Grobbelaar (134)].

The natural occurrence of the hydroxypipecolic acids has already been noted (see Table II and its accompanying references). The occurrence of the 5-hydroxy compound naturally suggests a relationship to hydroxylysine, similar to that which obtains for pipecolic acid and lysine, with the substance baikiain as a dehydration product. This admits of the conversion of the 5- to the 4-hydroxy compound. These steps seem inherently probable, but they lack proof. In view of the difficulty in accounting for the formation of hydroxyproline, it may well be that hydroxypipecolic acid also originates in some other way. Since hydroxyglutamic acid is not a direct precursor of hydroxyproline, in the rat at least [Benoiton & Bouthillier (113)], the oxidation leading to hydroxypipecolic acid may occur after, rather than prior to the ring closure.

In the synthesis of the azetidine ring, presumed to occur from α-γ-diaminobutyric or aspartic acids, only analogy can suggest the sequence, with the added evidence that all the postulated intermediates are known to occur naturally. Whilst there is evidence for α-γ-diaminobutyric acid in the free and combined states (209, 221, 222), its deamination product, aspartic-β-semialdehyde, is known in yeast [Black & Wright (90)] and arises during reduction of aspartic acid to homoserine (223). Homoserine itself, following its identification as a natural product by Miettinen *et al.* (224), is now well recognized on chromatograms [Berg *et al.* (225)].

Thus, cyclization by deamination and condensation may now be recognized in various series of compounds (Fig. 1). From the substituted glutamic acids one can visualize the possible origin of certain of the substituted prolines, though this is not based on proof [cf. 8 (Fig. 1)]. It may also be noted that the porphyrin ring of haemoglobin is known (96) to arise from δ-amino laevulinic acid and this, in turn, from glycine and "active succinate"; the same conversion to chlorophyll is being investigated in *Nicotiana* by Roux (226).

The hydroxypipecolic acids.—The critical identification of naturally-occurring 5-hydroxypipecolic acid rests on the work of Virtanen & Kari (217), who matched a natural product with a synthetic one obtained from King *et al.* (216).

In *Leucaena glauca*, Hegarty (227) has also recognized 5-hydroxypipecolic acid, and has matched it with the material previously recognized from the date and from legumes (9).

The synthesis of 5-hydroxypipecolic acid from hydroxylysine has been carried out by workers at the National Institute of Health [Cohen *et al.* (228)]. The products of this synthesis were resolved into two diastereoiso-

meric forms, the configuration of which was not exactly stated, nor were they resolved into the optically active forms. It is now known that the naturally occurring 5-hydroxypipecolic acid represents one of the four possible structures (289). This is pertinent since the hydroxyproline of *Santalum* is the allohydroxy-L-proline [Radhakrishnan & Giri (229)], whereas that from hydrolysis of protein is the hydroxy-L-proline.

Azetidine-2-carboxylic acid.—This substance was recognized by Fowden (66) as a brown spot after reaction with ninhydrin on chromatograms of *Convallaria.* When isolated, this substance gave rise to several others by acid hydrolysis. This led Virtanen & Linko (230), who had obtained a similar material from *Polygonatum,* to describe the substance as a curious twelve-membered ring yielding homoserine as one hydrolytic product. However, after the determination of a correct molecular weight and elementary analysis, the identity was apparent; and Fowden confirmed his identification by synthesis of the optically active form from L-α, γ-diaminobutyric acid. The identity of the naturally-occurring and the synthetic compounds was established by infrared comparison and other criteria (66, 67). In the Liliaceae however, it appears that azetidine-2-carboxylic acid is of rather widespread occurrence, for it appeared in about a quarter of the extracts made in a survey which represented seventeen genera (42).

Ports of Entry for Inorganic Nitrogen: Nitrate Reduction and Nitrogen Fixation

Nitrate reduction and nitrogen fixation have been treated previously in this series (2, 16, 19, 21) and more fully in other reviews (231, 232). They are also dealt with by Nason (233), McElroy & Spencer (234), and Burris (235) in the recent symposium on "Inorganic Nitrogen Metabolism." These subjects, therefore, need only brief mention.

Despite such hypothetical intermediates as nitramide and hyponitrous acid, referred to by Burris (235), and by Nason (233), the main attention is still focused upon nitrate, nitrite, hydroxylamine, and ammonia, and the separate enzymes which reduce these substrates. These enzymes are adaptive since they are lacking when ammonia or alanine is the sole source of nitrogen and are produced when the appropriate substrate is furnished [see Nason (233); McElroy & Carter (234); and summary by Glass (236)].

Reviewing the history of nitrate reduction, Nason (233, p. 111–12) has drawn attention to a paper, long overlooked, by Kastle & Elvove (237) which demonstrated, as early as 1904, the conversion of nitrate to nitrite by potato tuber extracts. Nason concludes that Kastle & Elvove were really studying an enzyme system of potato which is now known to catalyze the anaerobic oxidation of aldehyde by nitrate. Those substances which behaved as accelerators of nitrate reduction, are now viewed as electron donors to the nitrate.

The role of light and the involvement of manganese and molybdenum in the early steps of inorganic nitrogen metabolism have been discussed for

XUM

many years. These problems seem now to be resolved. Reduced TPN (*Neurospora*) or both TPN and DPN (soy bean) can act as hydrogen donors in nitrate reduction. The well-known reduction of pyridine nucleotides by the photolysis of water [Vishniac & Ochoa (238)] links light to nitrate reduction and accounts for earlier claims [cf. Nightingale (239); Burström (240)] that leaves in the light have a special ability to reduce nitrate. It can be shown that chloroplast grana transfer electrons to nitrate in the presence of nitrate reductase [Evans & Nason (241)] via TPN, which is continuously reduced to TPNH (the electron donor) by light. The nitrate reductase now seems to be a molybdenum flavoprotein, although it has not been purified to the point of crystallization nor obtained free from all other enzymatic activities.

Molybdenum as a component of nitrate reductase has been established for *Neurospora* [Nicholas & Nason (242)], soybean, and *E. coli* [Nicholas & Nason (243)]. According to Wolfe (244), molybdenum is concerned in *Anaboena* with synthesis of the reducing compounds rather than with reduction of nitrate per se. When either fungi or higher plants utilize nitrate as the nitrogen source, the specific requirement for molybdenum is greater than when they use ammonia. A molybdenum growth requirement, which is independent of nitrate reduction, also exists [Nason (233)].

The further reduction of nitrite and of hydroxylamine, still the favored intermediates, is also catalyzed by metalloflavoproteins. Mahler & Green (245) regard nitrite reductase and hydroxylamine reductase as mediated by a metalloprotein with unknown metallic content; nevertheless Nason (233) has drawn attention to the markedly stimulating effect of manganese upon both enzyme systems as isolated from the soybean. Nason regards these facts as support for the findings of Burström, Leeper, Hewitt *et al.*, who implicated manganese in nitrate reduction because nitrate accumulated in the leaves of manganese-deficient plants. Although the nitrite and hydroxylamine enzymes have not been as accurately characterized as nitrate reductase, they may well utilize manganese. Thus, molybdenum is implicated in the first step of nitrate reduction, manganese may still be implicated in the later steps. With a greater or lesser degree of specificity manganese has been implicated in other enzymes of nitrogen metabolism [e.g., in glutamyl transphorase (233a)]. A result of manganese deficiency is often the accumulation of amino acids, but full treatment of these effects is beyond the scope of this review.

Nitrate reduction via molybdenum-flavoproteins is not universal in plants, for in *E. coli* a cytochrome, b_1, may mediate the flow of electrons to nitrate (instead of to oxygen), as indicated by work ably summarized by Glass (236). Also, in *Pseudomonas*, the reduction of nitrate by nitrate reductase under anaerobic conditions is coupled to the oxidation-reduction of cytochrome *c*.

Nitrogen fixation.—Nitrogen fixation retains its intrinsic interest as well as its significance for the path of nitrogen more generally. From the clas-

sical work of Bortels on *Azotobacter*, molybdenum has been implicated in nitrogen fixation in this organism and in *Clostridium* and *Anaboena* [cf. Anderson (246)]. Fixation by *Rhizobium* was earlier restricted to active attached nodules, but it has now been consistently demonstrated with excised nodules [cited by Magee & Burris (247)]. The entry of nitrogen into organic combination may occur either via hydroxylamine (to form aspartic acid from oximinosuccinic acid) or ammonia (to form glutamic acid), according as the conditions promote one or the other of these steps. This disposes of any claim that either ammonia or hydroxylamine have to be the exclusive "alpha and omega of nitrogen metabolism." While Burris remains faithful to ammonia (because of the ready demonstration of its use as an exogenous source of nitrogen and the ease with which N^{15} from ammonia may enter into organic combination), nevertheless, he now recognizes the claims of hydroxylamine to be an intermediate (235).

Mutant strains of *Neurospora* are known which grow on ammonia without pyridoxine, but which require this vitamin in the absence of ammonia. Silver & McElroy (248) propose that the pyridoxine-requiring pathway, which bypasses ammonia, is a sequence of steps from nitrate through hydroxylamine, the oxime of pyridoxal phosphate, and pyridoxamine followed by group transfer of the amino group, thus regenerating pyridoxal. If the endogenous source of pyridoxine is blocked in a mutant, an external source of pyridoxine is needed. This line of evidence coupled with the detection of oxime nitrogen and of hydroxylamine reductase, strengthens the case for hydroxylamine as a possible intermediate, but, as Burris maintains, not to the exclusion of ammonia.

By the use of N^{15}, Burris and his collaborators have continued their elegant studies on the path of nitrogen in fixation and excretion, and consistently point to evidence regarded as compatible with "the ammonia hypothesis." The evidence is that the atom per cent of enrichment of nitrogen with N^{15} is not in the order hydroxylamine > oxime > amino acid > ammonia, as the hydroxylamine hypothesis requires, but favors the alternative ammonia hypothesis. Burris (235) stresses the evident toxicity of exogenous hydroxylamine and the consequential difficulty of making experiments with this substance. Thus (235, p. 332): "We frequently are asked why we do not make more extensive tests with N^{15}-labelled hydroxylamine. Experiments with labelled hydroxylamine are subject to the same difficulties as are those with the unlabelled material, namely, the difficulties from toxicity and decomposition of the compound." For example, Burris states that Novak & Wilson (249) found that a few parts per million of hydroxylamine initially stopped the growth of *Azotobacter*, but growth was resumed when the hydroxylamine was decomposed. The argument concerning the toxicity of hydroxylamine may seem to be a cogent one; it does not, however, dispose of the problem. Many normal metabolites and intermediates have different properties when supplied exogenously. Solutions, such as those known by actual analysis to be contained in *Valonia*, are externally toxic to the same cells; and, it is

said that the amount of potassium in the human heart, if instantly released into the blood stream, could kill a man! Therefore, a positive observation of the use of exogenous hydroxylamine would be meaningful; a negative conclusion does not eliminate hydroxylamine as an intermediate in amounts that would suffice for such a role.

The balance of evidence now seems to be that in the path of nitrogen from nitrate to amino acid, either hydroxylamine or ammonia, or both, can figure as intermediates; and the direction which the reactions take will be determined by the conditions.

The interpretation of nitrogen fixation, like all problems of intermediary metabolism, is complicated because the process occurs in cells whose complex organization is commonly disregarded. With great skill and technical ability, Burris (235) has marshalled the evidence as follows. *Azotobacter* will convert N_2^{15} into $N^{15}H_3$ which appears in the external medium. The N^{15} content of the NH_3 in the medium may reach a figure greater than the N^{15} content of the N in the cell, and in 45 minutes half the total NH_3 in the external medium may become labelled with N^{15}. The organism shifts easily and promptly from the use of N_2 to NH_3, and the compounds marked with N^{15} are similar whether the external source is N_2 or NH_3. All this evidence commands respect. But when Burris (235, p. 326) says: "This experiment constitutes direct evidence for the participation of ammonia as an intermediate in the nitrogen fixation process" we have to ask whether this really follows? How is the interpretation affected by the obvious facts of protein synthesis and breakdown in *Azotobacter* and by the fact that anabolism and catabolism inevitably proceed side by side in the cells? If one visualizes the *Azotobacter* cell, with its carbon passing through a rapid cycle of protein synthesis and breakdown localized in different phases of the cell, with the end products (CO_2 and NH_3) diffusing into the external medium, an equally good case can be made for the NH_3 as an end product rather than an active intermediate. The fate of an active intermediate in the anabolic phase can hardly be discerned by events in the catabolic phase of the cell, or in the external medium. This argument is not given to discredit Burris's view but to show that intermediary metabolism needs to be interpreted in a manner compatible with the heterogeneity of cells, and their separate phases and inclusions.

Ports of entry for nitrogen into organic combination.—Traditionally organic combination with inorganic nitrogen leads to the formation of amino acids or their amides. α-Ketoglutaric acid, oxaloacetic acid, and pyruvic acid are the favored acceptors for nitrogen leading to amino acids; and glutamine and asparagine are the two principal amides formed. According to Roberts *et al.* (30) this also applies to *E. coli* (30, p. 432). Direct detection of keto acids in plants now suggests that there may be other similar possibilities. If the condensation proceeds via glyoxylic acid it may well involve urea and lead to purine synthesis. The role of these N-acceptors implies reduction of nitrate prior to condensation. However, the now demonstrable occurrence

(117) and reduction (250) of nitro compounds in nature suggests the possibility that nitrate reduction may sometimes occur after organic combination by a direct nitration. This would bypass the keto acids as acceptors. The possibility that hydroxylamine may combine with carboxyl groups to form hydroxamic acids has previously been entertained (cf. 2). This occurs in enzyme-catalyzed reactions and is now believed to occur in yeast furnished nitrite to form acetyl hydroxamic acid (251), which was detected chromatographically by the use of a ferric chloride test. It is now claimed (251a) that most of the soluble nitrogen of nodules of white clover yields γ-aminobutyric acid on hydrolysis. While it has been presumed for a long time that ammonia is fixed in citrulline via the ornithine cycle, recent evidence gives this type of reaction a more general significance through the formation of carbamyl compounds (e.g., of aspartate or glutamate) from CO_2 and NH_3. These in turn donate their carbamyl groups and incorporate these through a variety of transfer reactions into other amino acids (17).

Nitrogen Metabolism and Other Physiological Functions: Protein Synthesis

Every discussion of nitrogen metabolism, classical or modern, culminates with the problem of protein synthesis and protein regulation. It is at this point that nitrogen metabolism must be related to respiration, cell division and growth, nutrition, and photosynthesis. As recently as 1953, Wood (19) summarized the approach which considers protein synthesis in terms of the external and internal variables which impinge upon it, and which seeks to stipulate concentrations of cell constituents or rates of their reaction which regulate the synthesis. Even early in this phase, physiological effects attributable to hormonal regulation were recognized by Chibnall (3).

The energy relations of protein synthesis, the elaboration of the organic acid cycle, and the role of the keto acids all compelled increasing attention to both respiration and protein synthesis. The next step was to visualize some mechanism by which respiratory energy might be used in synthesis. By 1947 (1) the idea that phosphate bond energy could contribute directly to peptide bond formation was already familiar, but the process was still essentially dependent on the cell as a whole: in fact, dividing cells by virtue of their self-duplicating properties seemed to be important centers for protein synthesis. Indeed, later work was required to re-direct attention to the protein synthesis in expanding cells (252); but the role of specific inclusions, mitochondria, and microsomes, in protein synthesis was already an active subject for speculation.

By 1950 (2) the study of protein synthesis in less organized systems was launched by the incorporation of radioactively labelled amino acids into protein, using animal preparations. This review ended on the note that when these techniques were applied to plants, they would give the direct approach to protein synthesis that was needed. However, the results obtained in the intervening years with noncellular preparations have been disappointing.

XUM

Attempts to study protein synthesis in simpler systems have taken several directions. First, virus protein has been recognized as a special case of protein which is synthesized and segregated from the rest of the cell. In this area the template hypothesis, the role of pentose nucleic acids, and speculations upon the molecular architecture of the synthesizing surface have been prominent. Secondly, direct attempts have been made to dispense with the organized cell of higher plants and to demonstrate synthesis in cell-free preparations. A third trend concerns the groups transfer reactions through which syntheses are conceived as originating in a few primary reactions which accumulate respiratory energy in simple compounds like glutamine. Following this, other reactions with lower energy requirements complete the synthesis of complicated molecules via group transfer reactions in which all, or parts, of the primary molecules freely rearrange or combine. Such ideas have led to attempts to formalize the reactions of protein and nucleic acid synthesis [Dounce (253, 254)] and, premature as this may be, to relate such ideas to other physiological functions (e.g., ion accumulation and respiration) which are linked to protein synthesis and breakdown [Steward & Millar (255)]. The latter scheme attempted to show how a principal obstacle to the role of the γ-glutamyl transpeptidation system of Hanes, Hird & Isherwood (256) in protein synthesis might be overcome. It is visualized that the variety of γ-glutamyl peptides formed by transpeptidation represent carriers of amino acid which are hydrolyzed at the template surface, enabling α-linkages to be formed. Thus, in a speculative way the template hypotheses and the group transfer reactions may be brought together. Since the group transfer reactions were the principal topic of a recent review [Webster (16)], they will not be dealt with further.

Cell inclusions and particulate preparations.—It is pertinent to analyze the progress made by the use of cell free preparations. In a comprehensive review by Lindberg & Ernster (194), largely concerned with animal cells, the relation of mitochondria and microsomes to protein synthesis is indicated by the following salient passages:

> An important part of the cytoplasm, up to 50 per cent of its total mass, consists of particulate elements. Some of these, the mitochondria, are visible under the microscope and are usually rod shaped; while others, the microsomes are of submicroscopic dimensions . . . . The mitochondria contain all the iron porphyrin respiratory enzymes of the cell; while the microsomes are bearers of the greater part of the cytoplasmic nucleic acid . . . . The RNA-rich submicroscopic particles of the cytoplasm are believed to be mainly concerned in protein metabolism . . . . Isotope experiments ***in vivo*** show that the renewal of RNA is most rapid in the nucleus, while the turnover of proteins is most vigorous in the microsomes. The microsomes are, therefore, supposed to be the organs in which the protein synthesizing activity of the nucleus is brought to completion . . . . The ribonucleoproteins deriving from the nucleolus are probably the active principles for the protein metabolism of the microsomes . . . . The mechanism of this activity presumably consists in transpeptidation reactions . . . . The energy for this process is provided by the mitochondria, perhaps in the form of certain "key peptides" containing γ-glutamyl groups . . . . The micro-

somes . . . are in all probability involved in the production of the specific proteins of the cell, including the enzymes.

These quotations may be accepted as showing the broad background of knowledge in this field, but recognizing that differences between mitochondria and microsomes may be difficult to draw sharply.

Yemm (257) points to difficulties that arise if ability to synthesize protein is restricted to microsomes, because N^{15} from NH_4Cl is very rapidly dispersed into all cellular inclusions, suggesting that primary synthesis of new protein may occur in nuclei, plastids, and mitochondria of active, young seedlings.

It is against this general background that the work on protein synthesis in noncellular preparations made from higher plants should be discussed. The work of Webster is important because it deals with preparations made from higher plants.

The first serious claim that plant mitochondrial preparations could incorporate amino acids into protein was made as recently as 1954 (258). In this sort of work the "synthesis" of protein is traced by the C^{14} from amino acids which appears in the protein moiety of the preparation. The enzymatic preparations were homogenized tissue from young bean hypocotyls subjected to controlled centrifugation resulting in relatively uniform particles. By 1955, however, the claim was extended to imply that similar preparations were capable of a net synthesis of total protein (259).

At the presentation of the 1955 paper, a definite claim was made that the cell-free preparations from pea seedlings synthesized new protein, to the stated extent of about 20 per cent of that originally present. At this point, Webster believed that protein synthesis required ATP, Mg ions, a mixture of amino acids, and a certain amount of ribonucleic acid. In an earlier work which appeared somewhat later (260), no direct mention was made of net protein synthesis, and the nucleic acid was replaceable by purine and pyrimidine bases. Still another paper (261) referred to a requirement for ATP and Mg for the incorporation of glutamic acid into protein of pea seedling and other homogenates. In this paper the simultaneous presence of other amino acids promoted the incorporation of C^{14}-glutamate while the individual acids were not effective. The alleged synthesis, or incorporation, was measured by determining C^{14} in the protein from C^{14}-glutamic acid. As late as 1956 (262), Webster has claimed "effects of nucleosides and their derivatives on protein synthesis in cell free extracts of pea roots." However, work in this area encounters several difficulties. First, critical and absolute sterility must be maintained throughout all the experiments, since bacteria are proverbially active agents for protein synthesis. Secondly, when the measurements consist only of total C^{14} it becomes imperative to remove all substances attached to the protein to insure that the C^{14} so measured is not merely adsorbed by, rather than incorporated in, the protein. These difficulties are very real ones. While

XUM

Webster has reaffirmed his original (259) claims to net protein synthesis in subcellular preparations in general terms (262), no analytical data are supplied in these statements. However, Webster does not believe that bacteria could be responsible for his results (262a).

The principal claims are that these preparations form new peptide bonds because C^{14}-labelled amino acids are said to be "incorporated" in the protein. Again the evidence of incorporation depends only on the measurement of C^{14}, and the amounts so measured are often small relative to the activity supplied. Direct isolation or detection of the incorporated amino acids which contain the radioactivity was not done. [An interesting device to overcome the difficulty of establishing synthesis of a minute amount of new protein is to demonstrate the incorporation of C^{14}-amino acids in an adaptive enzyme, not previously present in the system, as in the works of Spiegelman *et al.* (263).] The difficulty is to assess what "incorporation" really means.

However probable these ideas may seem to their advocates, they still lack absolute proof. Before this idea can be accepted, all possibilities that the C^{14}-labelled substrates are merely attached to, or loosely bound upon, the protein need to be rigorously excluded. While it is true that the preparations are washed and dialyzed in various ways, the difficulty of complete removal of material adhering to the protein is quite obvious. Since similar preparations from beans (264) rapidly synthesize glutathione, it is conceivable that oxidative formation of disulphide bonds, not necessarily enzymatic, could bind glutathione (or any other cysteine peptides so synthesized) to the thiol groups in the proteins of the particulate preparations. As long as any such alternatives are in accord with the data (notably the stimulus of mixtures, containing among other substances cysteine and glycine, to incorporation of C^{14}-glutamate) the validity of true protein synthesis requires more rigorous proof. [For a discussion of the difficulties of interpreting amino acid incorporation experiments made on animal cells and bacteria, reference may be made to Tarver (265).]

An investigation by Stephenson, Thimann & Zamecnik (266) is relevant here. This work demonstrated the passage of C^{14} from the carboxyl of exogenous amino acids into the protein fraction of leaf disks and of fractionated cell-free preparations. The cell free preparations incorporated C^{14} from amino acids at their initial rate for about thirty minutes, and are said to use these sources more readily than $C^{14}O_2$. Activity continued for about an hour, and the authors claim only about 0.3 to 0.6 per cent replacement of a protein amino acid by the carbon of its C^{14}-counterpart. The isolated chloroplast fraction was the most active and this, like the leaf, responded to light and oxygen, but not to added ATP. The isolated microsomes had least activity, although in the intact leaf, the C^{14} entered the microsomes first. By various washings, precautions were taken to distinguish combined C^{14} from superficially bound compounds, and antibacterial agents were used to eliminate the effect of bacteria. Whereas the richest incorporation into protein was in the TMV fraction of infected tobacco leaves, the virus protein added to

cell-free preparations did not incorporate C^{14} from $C^{14}OOH$ of amino acids. The authors recognize that the intact cell is needed for TMV protein synthesis—a conclusion that might well have been extended to the *de novo* synthesis of any protein.

Whatever the final outcome may be, the following facts are now clear. No cell-free preparation yet prepared from higher plants compares, even remotely, with the intact cell system in synthesis of new protein, if rigorous precautions to exclude all possibilities of contamination with microorganisms are adopted. This suggests that in the intact cell the mechanism of protein synthesis *in vivo* still involves the cellular organization over and above the attributes of individual particules such as mitochondria and microsomes.

Protein metabolism of intact cells.—In this context a study made by Steward, Bidwell & Yemm (158) is relevant. This investigation employed aseptic tissue cultures, either growing rapidly under the stimulus of added coconut milk or growing very slowly in its absence. The cultures were treated externally with uniformly labelled C^{14}-glucose, C^{14}-γ-aminobutyric acid, or C^{14}-glutamine. By chromatographic, radioautographic, and specific activity determinations, the path of the C^{14} was traced through the alcohol-soluble fraction of the tissue, the hydrolysate of the alcohol-insoluble residue containing protein, and into the respired carbon dioxide.

The specific activity data showed concurrent, but quite different reactions in the cell, involving the same substrate; these must be located in geographically separated phases, or compartments. In these separate phases metabolic pools containing the same substance may preserve separate identities. Two such broad phases or compartments are visualized: one into which exogenous solutes enter and in which anabolic reactions and protein synthesis predominate; and another, supplied endogenously from protein breakdown, in which catabolic reactions predominate.

Free amino acids of the cell were not directly incorporated into the protein synthesized, for this received its C^{14} label much more directly from sugar than from amino acid. If amino acids are in fact intermediates in the conversion of the carbon of sugar to protein at the site of synthesis, they cannot freely mingle with the free amino acids of the cell. The experimental data become intelligible if it is recognized that, for at least a part of the protein, protein turnover occurs and the growth factor stimulates this turnover as well as net synthesis. Thus, the events at the seat of protein synthesis cannot be segregated from those occurring elsewhere in the cell, for the pace of synthesis is determined by the operation of a cyclical system in which the path of carbon into protein is mainly via sugar in the anabolic phase, and into the respired carbon dioxide via the products of protein breakdown in the catabolic phase. On present knowledge, these phases cannot be strictly identified with mitochondria, microsomes, nuclei, or other cellular components. [See Figure 1 of Steward, Bidwell & Yemm (158)]. Hence rigorous separation of the cell into its graded cytoplasmic particles or inclusions could well isolate parts of this cyclical system, but simultaneously rob it of any chance

XUM

of operating in a continuous manner. Following Smuts' doctrine of "holism" the cell is indeed more than the sum of its parts in respect of protein synthesis.

This picture of protein synthesis in the intact cell is not compatible with simple condensation of the free amino acids of the cell, but it is more compatible with the idea that carbon sources from sugar and nitrogen groups transferred from the most effective donors (glutamine, glutamic acid) are condensed at a site which provides the appropriate template and energy, but which, for the most part, precludes the direct participation of the free amino acids (e.g., glutamic) of the cell. Only a specific protein moiety, which seems incapable of breakdown and turnover, seems to incorporate amino acids, especially proline and hydroxyproline, directly from the main body of the cell. These experiments greatly extend certain general ideas which were being advanced by Steward & Street (1) in 1947 to account for the relations of protein synthesis to respiration and the effects of different variables upon these processes.

Other examples are now available which indicate more ready incorporation of the carbon of glucose than of the free amino acids of the cell into protein. Bidwell, Krotkov, & Reed (267) furnished wheat leaves with C^{14}-labelled sugar and glutamine. In the dark, free glutamic acid was labelled much more readily from glutamine than from sugar, but the protein glutamic acid was much more heavily labelled from the sugar than from glutamine or glutamic acid. Roberts *et al.* (30) found that C^{14}-labelled arginine, the leucines, and proline were incorporated into protein without dilution and with little or no dispersal of their radioactivity; however, when glutamic acid, aspartic acid, alanine or threonine were furnished, the radioactivity was much dispersed, suggesting prior incorporation of their carbon in a metabolic pool before its incorporation into protein. In the protein, proline and the basic amino acids were actually more radioactive than glutamic acid and aspartic acid, even when they were formed from supplied glutamic acid, thus showing that in the protein the carbon of proline and the basic amino acids arose more directly from glutamic acid than did the carbon of glutamic acid itself.

Later experiments with *Torulopsis utilis* by Cowie & Walton (268) lead these authors to a somewhat similar position via a different experimental route. They visualize the cell as containing pools of amino acids which are protein precursors, but these pools are not of free (soluble) amino acids but compounds bound to, or associated with, some macromolecular "R-group." The free or soluble amino acids and those in the medium are not directly accessible at the site of protein synthesis, except as they are first "segregated" by their affinity for one or another "R-group." The synthetic amino acid pool is not here regarded as a mere mixture of "free amino acids" of the cell, but it represents "a direct intermediate in the synthesis of protein from exogenous fructose"; and interconversion of amino acids in the "metabolic pool" may occur without their removal from this complex. On this view

exogenous amino acids may compete with their counterparts formed endogenously from fructose and interfere with the direct flow of C^{14} from fructose via the "amino acid-R complex" into protein. Finally, "protein synthesis in *T. utilis* and *E. coli* appears to proceed by some process wherein all of the peptide bonds linking together the amino acids into protein are formed in one event."

Synthesis of virus and virus protein.—The interest in virus synthesis, as a special case of protein synthesis, continues. Earlier reviews have dealt with this problem [Bawden & Pirie (269); Steward & Thompson (24)]. Virus proteins are still among the most completely characterized plant proteins [see Table I of the review by Knight (270)], and for the terminal amino acids in the chain see Niu & Fraenkel-Conrat (271). End group hydrolysis by carboxypeptidase, showing threonine on polypeptide chains of tobacco mosaic virus, leads to the idea of about 2900 fundamental protein building units of 17,000 molecular weight in a virus protein of the order of 5×10^7 molecular weight.

The use of infectivity as a quantitative assay of virus protein being synthesized is somewhat complicated, because virus-infected plants also synthesize a nucleic acid-free, noninfective, protein ("abnormal protein"). This appears to be virus protein, based on its amino acid composition, on immunological properties, and on electron microscopy [Delwiche *et al.* (272)].

The so-called reconstitution of virus from its separated nucleic acid and protein moieties has a special bearing on the problem of protein synthesis. The ability to separate and reconstitute so complex a molecule as a nucleoprotein represents a major advance. Such claims have been made both on the basis of infectivity and electron microscopy [Fraenkel-Conrat & Williams (273)], on the production of "hybrid" virus with "unnatural" combinations of nucleic acid and protein [Fraenkel-Conrat (274)], and on the evidence of infectivity alone [Lippincott & Commoner (275)]. However, if Gierer & Schramm (276) are correct and infectivity may be carried by protein-free nucleic acids, much of the evidence of *in vitro* reconstitution is meaningless. Apart from such considerations, the protein synthesized in virus-infected plants represents one of the most directly demonstrable examples of protein synthesis.

The experiments on reconstitution bear on the somewhat similar problem of amino acid incorporation. *In vitro* separation and recombination of nucleic acid and protein, in a manner which retains the specific structure on which infectivity depends, approaches in complexity the removal of amino acids from protein and their replacement as in "incorporation."

The views of virus synthesis which regard it as forming at special sites in the cell where protein and nucleic acid combine, still leave the primary problem of the synthesis of the protein moiety, both its smaller and larger units, to be explained. It is noteworthy, however, that in both these fields, i.e., *in vitro* protein synthesis and *in vitro* virus reconstitution, the first optimistic claims are now being qualified.

XUM

Respiration.—Despite the obvious effects of nitrogen nutrition (NO_3; NH_3; amino acids; etc.) on respiration, classical concepts of the respiratory process have rarely invoked the nitrogen compounds to any important extent. Glycolysis and the Pasteur effect, Krebs' cycle, terminal oxidation and the "direct oxidation pathway" all seem to pursue their relentless paths to carbon dioxide, without recourse to nitrogen compounds, except as enzymes and co-enzymes. Chibnall (3), Gregory & Sen (277), and later Steward *et al.* (278), and Steward & Street (1) all in various ways departed from this simple view and invoked direct relationships between respiration and nitrogen metabolism, crediting alternating cycles of protein synthesis and breakdown with a major role in the path of carbon toward CO_2. Such ideas were summarized in 1947 (1), a regulatory role being attributed to carbon dioxide pressure inasmuch as it diverted the Krebs' cycle from the use of carbohydrate to the use of the deaminated products of amino acids, when the nitrogen of the latter was being used in protein synthesis. How does this idea stand today?

The general scheme of Steward & Street, 1947, (1) received some later justification in that $C^{14}O_2$ is fixed by potato slices and the carbon appears mainly in the five carbon compound glutamine [Steward & Pollard (8)]. The study by Steward, Bidwell & Yemm (158) is again compatible with, and actually extends, the view summarized above, for, in the operation of the "protein cycle," sugar furnishes the ultimate source of carbon which then becomes available for respiration after prior incorporation into protein. The fact that C^{14}-γ-aminobutyric acid gives rise readily to labelled glutamine in carrot tissue cultures and both C^{14}-γ-aminobutyric acid and C^{14}-glutamine give rise readily to labelled CO_2, while C^{14}-sugar tends to label CO_2 progressively, only as it labels the protein, is consistent with the view that much of the carbon of sugar needs to be built into protein before it is respired.

The respiratory use of the products of protein breakdown was demonstrated in senescent wheat leaves, which had been so starved that exogenous sugar could not be respired [Krotkov (279)]. Protein breakdown occurs in pea plants in the dark and amino acids accumulate, with the exception of aspartic and glutamic acids, which, though products of breakdown, seem to be re-used (41). Working with *E. coli* Roberts *et al.* (30) also conclude that there must be an alternate route to the familiar Krebs' cycle, for (30, p. 426) "it is clear that some reaction sequence other than the Krebs' cycle must be responsible for the large quantity of CO_2 produced." It is also stated (30, p. 427) that "when nitrogen is omitted from the medium the rate of CO_2 production drops to roughly one half of the rate during growth." These observations suggest that a comparable protein cycle exists in *E. coli*.

The idea of a protein cycle in respiration also explains some otherwise inexplicable results obtained when C^{14}-glucose was supplied to wheat leaves [Vittorio *et al.* (280)]. While the addition of C^{14}-glucose to wheat leaves increases the total respiration, surprisingly enough the additional carbon dioxide is mainly unlabelled and must, therefore, come from another source,

presumably the catabolic phase of a protein cycle accelerated by the added sugar. The protein breakdown products including CO_2 would be initially unlabelled, but would become progressively labelled as the protein itself incorporated C^{14} from glucose.

Physical separation of anabolic and catabolic nitrogen metabolism in distinct phases in the cell, with restricted exchange between them, can explain a number of otherwise puzzling results. Roberts *et al.* (30) have shown that C^{14}-labelled threonine and lysine, supplied exogenously to *E. coli* passed through different reactions than endogenous threonine and lysine. The comparative unavailability of early products of photosynthesis for respiration suggested that the Krebs' cycle is inoperative in light [Calvin & Mancini (281)], yet it clearly can operate in the light [Bidwell *et al.* (267)]. These two facts may be explained if the exogenous substrates or the early products of photosynthesis enter first a phase or reaction sequence different from their endogenous counterparts. The need of prior utilization of such compounds, through the protein cycle, before they become directly available for respiration still seems the best explanation of such results [Steward & Thompson (24)], especially in view of the known stimulus to protein synthesis in the leaf in the light.

Biosynthetic attempts to label asparagine strongly with C^{14} have proved unexpectedly difficult [Wilson *et al.* (282)]. This suggests that there is not always a ready and direct passage of carbon from sugar to asparagine even in leaves that store much of it (e.g., lupin, broadbean, and asparagus), but that the carbon reaches asparagine much more indirectly (e.g., after prior incorporation in protein).

The existence in oat seedlings of two types of L-glutamic acid dehydrogenase, one soluble and DPN dependent, the other insoluble, associated with cytoplasmic inclusions and DPN independent, as shown by Rautanen & Tager (283), is also compatible with different types of glutamic acid metabolism in different cellular compartments.

Photosynthesis and leaf cell proteins.—The green leaf in the light has long held a special place in protein metabolism, because photosynthetic products may be directly used as carbon sources in nitrogen metabolism. Equally, the leaf proteins (present mainly in chloroplasts) have a special interest. In work summarized by Wildman & Jagendorf (20), and later by Steward & Thompson (24), Wildman *et al.* (284) regarded the leaf proteins of dicotyledons as consisting of fractions divisible with the ultra centrifuge. Fraction 1 was a homogenous heavy protein containing ribonucleic acid, believed to be a cytoplasmic constituent, and having phosphatase activity and containing bound auxin. Fraction 2 was believed to be a heterogenous protein fraction composed of lighter proteins with various enzymic properties. The nature of fraction 1 now needs to be re-evaluated, for it can apparently be prepared from different leaves and obtained pure and free of nucleic acid. It is shown to come largely, if not exclusively, from the chloroplasts and to possess uniquely the properties of the enzyme of Horecker *et al.* (285) which carboxylates

XUM

ribulose diphosphate. Phosphatase activity and bound auxin are no longer believed to be main characteristics of fraction 1. However, a newly discovered RNA-rich protein fraction of leaves and seedlings, having a molecular size comparable to virus protein, readily decomposes, and this may have confused the earlier picture of the leaf proteins [cf. work of Lyttleton (286) on clover, and of Lyttleton & Tso (287)].

Arnon (288) and his collaborators now stress that chloroplasts generate ATP without oxygen, except that which comes from the photolysis of water. Photosynthesis through photosynthetic phosphorylation, obtainable even with shattered chloroplasts, could, therefore, drive protein synthesis more directly than the mere supply, via respiration, of carbon sources. This type of effect is invoked to explain recent work from Thimann's laboratory (266) and to explain the efficacy of chloroplasts in the light to incorporate C^{14}-amino acids into protein.

Future trends.—Evaluation of past achievement invites comment on future trends. Much chemical description of the nitrogenous metabolites is still required, though identification alone is not enough, for known metabolites and the metabolism to which they lead have been ignored because they did not fit conventional ideas (e.g., urea and the ureides). The powerful new techniques (chromatography, radioautography) will need to be applied critically, for there is still no substitute for isolation and rigorous identification of new natural compounds. Nitrogen metabolism is only a part, though a fundamental part, of the whole metabolic pattern, and it needs to be continually re-evaluated in its relation to other physiological functions. The range of unexpected keto acids and compounds that can act as nitrogen group acceptors presents a major problem in the linkage of nitrogen metabolism with carbohydrate metabolism and respiration. Intermediary nitrogen metabolism faces problems concerning the cellular inclusions and the extent to which these are autonomous organs of the cell or, by contrast, only fulfill their role by their part in the smooth operation of machinery that depends upon the cellular organization. Because plants achieve protein synthesis from the fewest and the simplest substrates, they still focus attention upon this paramount, but unsolved, problem in an especially challenging manner.

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PHOTOCHEMISTRY OF CHLOROPHYLL[1]

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Several of the recent review articles on photosynthesis in this series have given considerable attention to research results in the general field of chlorophyll photochemistry [Lumry *et al.* (1); Vishniac (2); Duysens (3)]. The last volume of the monumental monograph by Rabinowitch, which appeared during the summer of 1956, not only completes the original plan with its chapters on kinetics of photosynthesis, but also provides addenda to the earlier volumes which bring more or less up-to-date the topics treated previously in the monograph, including photochemistry of dyes in general and of chlorophyll in particular (4). A further summarizing work is the record of the symposium on photosynthesis held at Gatlinburg in October, 1955, under the auspices of the National Science Foundation. This volume, scheduled for publication in 1957, contains contributions from most of the American and some of the overseas investigators in the field of chlorophyll photochemistry (5). It thus seems unnecessary in the present article to present a comprehensive survey. Instead, several areas of current research interest will be reviewed with particular emphasis on developments during 1956, and on some earlier developments that have not previously been reviewed in this series. The first part of this chapter will deal with properties of chlorophyll *in vitro*, and the second part will treat some of the physical chemical aspects of excitation and photochemical sensitization in the green plant. The entire field of chloroplast reactions is being omitted here because of the separate article in this volume by Clendenning on the biochemistry of chloroplasts (6).

Chlorophyll *In Vitro*

Spectroscopy.—The importance of precise information about the absorption and emission spectra of chlorophyll cannot be overestimated. First of all, spectroscopy is the most commonly used analytical technique in the detection of chlorophyll reactions. It has become apparent in the past several years that light absorption must be employed with the greatest caution as a criterion of reaction. The absorption spectrum is not as sensitive to chemical change as was once thought and further, a variety of chemically modified forms of chlorophyll have very similar spectral features. Secondly, emission spectroscopy is one of the most useful tools for elucidating the kinetic limitations of photoexcited chlorophyll in terms of lifetime and energy content. Thirdly, the study of both kinds of spectra leads to rewarding information about the relationship of a chlorophyll molecule to its environment such as orientation in grana or other condensed phases, interaction between chloro-

[1] The survey of literature pertaining to this review was concluded in November, 1956.

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phyll and its substrate, and mode of transfer of excitation energy from the locus of original absorption to the site of chemical reaction.

The electronic absorption spectrum of chlorophyll in common organic solvents is by now well established, and should be easily reproduced since the development of methods for obtaining crystalline preparations by Jacobs, Vatter & Holt (7). These crystals, of either chlorophyll *a* or *b*, show characteristic x-ray diffraction patterns and possess amazingly high stability, retaining their spectral purity at least six months at room temperature in the dark. Smith & Benitez have redetermined the absorption spectra of ether solutions of carefully purified preparations of chlorophylls *a*, *b*, *c*, *d*, bacteriochlorophyll and their pheophytins (8).

Matlow (9) has made a theoretical molecular orbital calculation of the structure of porphin and dihydroporphin. The latter compound, a prototype for the chlorophyll molecule, had not been studied in earlier theoretical studies of more symmetrical porphyrin types. Matlow took into account the different electronegativities of the two types of central nitrogen atoms, those bound to hydrogen and those unbound. For porphin the two main peaks are predicted at 632 and 511 mμ. For dihydroporphin the main peaks are predicted at 651 and 464 mμ, with doublet separations of the two bands in the order of 11 and 6 mμ respectively. For both these molecules, estimates were made of the relative energies of isomeric forms differing in the distribution of the central two hydrogen atoms among the four pyrrole nitrogen atoms. The estimates of the energies of isomerisation, about 1 or 2 kcal. per mole for dihydroporphin, although crude, are of the right order of magnitude to account for the observed isomerisation equilibria of chlorophylls and chlorophyllides at room temperature. Freed had previously suggested such a a model for this isomerism, with the obvious substitution of magnesium-nitrogen bond localization for the hydrogen-nitrogen case considered by Matlow (10).

Holt has recently reported a new study of the infrared spectrum of chlorophyll and some of its derivatives (11, 12), to supplement the earlier infrared spectroscopy by Weigl & Livingston (13). These studies provide an interesting insight into the chemical behavior of ring V. This is the highly strained cyclopentanone ring containing a carbonyl at C-9 and the α-carbon of a substituted methyl acetate at C-10. The other members of the ring belong to the basic porphyrin framework. This ring, because of its high degree of strain, its known ease of enolization and oxidation, and its essential difference from the basic porphyrin pattern, is commonly viewed as the site of sensitization in chlorophyll reactions. Allomerization definitely produces an ether linkage at C-10 and not a free —OH group, at least in nonbasic solvents. A raising of the C=O stretching frequency in allomerized material was interpreted as evidence for oxidative rupture of ring V followed by subsequent lactonization between carbons 9 and 10. Holt interprets a band near 1660 mμ as a modified carbonyl stretching frequency indicative of hydrogen bond formation between the enolized C-9 and the ester carbonyl attached to C-10.

This absorption appears in crystals or in carbon tetrachloride solutions of chlorophyll *a*, but not in carbon tetrachloride solutions of pheophytin or chlorophyll *b*. Apparently the formyl group of the latter compound serves as an electron sink which reduces the electron density at the C-9 bond to oxygen; and magnesium has the opposite inductive effect. This band at 1660 mμ is apparently the one attributed by Weigl & Livingston to the —C=C— group in phytol, which in their work did not appear in all the phytyl-containing derivatives.

The flash spectroscopy of chlorophyll, first reported just a few years ago, has been the subject of very active current investigation. The importance of this work is in the definitive characterization of the electronically excited metastable state, postulated as the intermediate in chlorophyll sensitized reactions. The technique is to illuminate a chlorophyll solution so strongly as to force over 80 per cent of the molecules into the metastable state. Experimentally, such light intensities can be achieved only by the use of short flashes no longer than 100 μsec. While the molecules still retain their excitation, a measurement of the absorption spectrum of the metastable state is made with a much weaker analytical light beam. Livingston (14) and Linschitz (15, 16) and their co-workers observed the new absorption spectrum together with the kinetics of its rise and fall by recording on an oscillograph the intensity of the transmitted monochromatic analytical beam. Livingston, in addition, employed the spectrographic technique of recording the entire absorption spectrum by pulsing an analytical beam of white light through the sample and then into the entrance slit of a good spectrograph (17).

In order to secure reliable results, it is necessary to generate flashes of reproducible intensities. Also the flashing times should be short in comparison with the decay time of the metastable state, so that all molecules reach their state of excitation at the same time. In the earliest measurements, these criteria were not always met; as a consequence there were a number of anomalies in the decay kinetics, with the suggestion that a single metastable state was not sufficient to account for the results. In recent experiments with flash excitations of only a few microseconds duration and with elaborate baffles to keep traces of scattered light originating from the flash from entering the measuring photocell, Linschitz has succeeded in observing very reproducible curves that require only a single metastable state for their interpretation in the case of chlorophyll solutions in pyridine and in alcohol. All flash experiments showed that the metastable state has decreased absorptions at the wavelengths of the red and blue maxima of the normal chlorophyll, whether *a* or *b*, that the absorption between these two wavelengths is enhanced, and that absorption is increased in the far red above 680 mμ. The further details of the spectra are, at the time of this writing, still open to some question. Livingston reported maxima in the absorption of the metastable species at 460 mμ for chlorophyll *a* and at 430 and 485 mμ for chlorophyll *b* in benzene. Linschitz reported a shift of absorption to around 525 mμ for both chlorophylls, a wavelength at which Livingston observed increased

absorption but no peak. Again at about 700 mμ Linschitz reported an absorption peak, whereas Livingston reported only slightly increased absorption. It may be that the two sets of experimental results are not mutually contradictory. Livingston's spectrographic technique provides a continuous scanning of the visible spectrum and probably gives a fair picture of the general shape of the curve. Linschitz made observations at only selected wavelengths and his results, although potentially more accurate at any individual wavelength, require the collation of many experiments for proper formulation of the complete spectrum. As the experimental techniques are being refined in both laboratories, we can look forward to the final classification of these spectra within the next year. These spectral details have many implications for photosynthesis, where correlations might be attempted with the reversible enhanced absorption in illuminated chloroplasts at 515 mμ (see below). Franck's newest two quantum excitation hypothesis would require not only the existence of an enhanced absorption by metastable molecules in the far red but also the attainment by this absorption peak of an extinction coefficient approaching that of normal chlorophyll at its red absorption maximum. Further development of the flash spectroscopic technique should allow the direct study of the reaction of the metastable state with specific substrates.

The first flash experiments of Livingston & Ryan suggested that the long-lived excited state might consist partly of an intramolecularly changed species and partly of radical or ion products resulting from the bimolecular reaction of photo-excited chlorophyll with solvent or with an adventitious impurity in the solvent (14). This second possibility was in keeping with the previously observed kinetics of the small (about 1 per cent) reversible bleaching of chlorophyll under continuous illumination. Another complicated feature of the flash work was reported by Livingston in connection with the reversibility of the flash bleaching. Preliminary results suggested that the metastable state reverted to the ground state not only by a unimolecular process but also by collision with normal unexcited chlorophyll molecules; but Livingston now urges caution in such an interpretation of these results. Subsequent improved experiments indicate that metastable chlorophyll, like photoexcited states of other conjugated molecules that have been studied, reverts to its ground state independently of impacts with other unexcited molecules. Linschitz and Livingston both agree, in their preliminary reports, that the lifetime of the metastable state is in the millisecond range in normal solvents at room temperature, where the chlorophyll concentration is 10^{-6} to $10^{-5}M$. Both agree also that the flash bleaching occurs efficiently in very dry hydrocarbon solvents and Linschitz has observed bleaching with allomerized chlorophyll.

These latter experiments seem to rule out the possibilities that the bleaching requires solvation at the magnesium atom (15) or enolization at carbons 9 and 10, and suggest that the bleached state is an intramolecularly rearranged form. Although the proof is not conclusive, the metastable state

seems likely to be a triplet state. The chemical consequences of this inference derive from the high reactivity of the two unpaired electrons appearing in triplets. In this connection the recent work of Evans might be cited, in which a very sensitive magnetic balance was used to confirm the paramagnetic, and hence, triplet character of the photo-excited metastable state of fluorescein and a few other conjugated molecules (18). For triphenylene, the disappearance of the photomagnetism followed the same time course as the decay of phosphorescence. Since Lewis and Kasha's original identification of the phosphorescent state of conjugated molecules as the triplet state, it has been increasingly popular to accept the triplet identification of the photo-excited metastable state, even if phosphorescence has not been observed [Kasha & McGlynn (19)]. Evans' recent work is the best experimental confirmation of this view for any particular substance, and it is agreeable to have his data on record after the many unsuccessful attempts in recent years to observe photomagnetism by the electron spin resonance method. Such negative results can be interpreted either by the nonexistence of a triplet state or by the unsuitability of this method for such substances, for which many reasons can be advanced. Whatever the nature of the metastable state, the importance for chemistry is that it lives at least 100,000 times as long as the fluorescent state. The added lifetime provides time for the excited molecule to wait, if necessary, for a reaction substrate to diffuse up to it. The metastable state was first proposed on kinetic grounds to account for the observation that chlorophyll fluorescence was not weakened during the efficient sensitization of thiourea oxidation [Gaffron (20); Franck & Livingston (21)].

In addition to the tabulation of excited electronic states to which the metastable state may be raised, which we now know at least partly from flash spectroscopy, it would be very useful to know the amount of energy stored in the metastable state with respect to the ground state. Such information would bear directly on the question of the maximum energy efficiency of photosynthesis. More properly behaved dye substances phosphoresce at low temperatures with a delayed emission spectrum displaced toward longer wavelengths with respect to normal fluorescence. The energy corresponding to this low temperature phosphorescence is the energy storage in the metastable state and is the energy available for sensitized photochemistry. In spite of the repeated efforts of at least half a dozen investigators, phosphorescence of chlorophyll *a* has never been observed. Becker & Kasha were the latest to confirm a very feeble phosphorescence of chlorophyll *b*, using a glassy solvent of ether, isopentane, and alcohol at liquid nitrogen temperature (22). Becker reported that the phosphorescence has a lifetime of about 10^{-4} seconds (23). If this phosphorescence represents the decay of the same state as observed in flash spectroscopy, it is difficult to see why the lifetime should not be appreciably lengthened on cooling from room temperature to 77°K. This is one of the many anomalies that occur in chlorophyll photochemistry that do not occur in simpler prototypes. Since the *a priori* prediction of the transition probability of chlorophyll *b* phosphorescence is unsatis-

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factory, one can simply accept the absence of phosphorescence of chlorophyll *a* philosophically for the time being without having to overthrow his belief in the otherwise well-established metastable state. It seems to be unnecessary to go so far as Becker has done (23) in suggesting that in photosynthesis the primary chemical energy transfer step occurs at a complex of a substrate with chlorophyll *b* rather than *a*. Such a proposal contradicts a wealth of experimental and theoretical work on the energy transfer from chlorophyll *b* to *a* [Lumry *et al.* (1)].

Fluorescence.—A reinvestigation of the absolute yield and lifetime of chlorophyll fluorescence has been reported within the past year. This information is of considerable importance to photosynthesis in that it defines limitations on the use of the energy stored in the fluorescent state either for direct photochemical reaction or for transfer to other pigment molecules. Latimer, Bannister & Rabinowitch (24) measured the fluorescence yield of chlorophyll by the integrating sphere technique previously used by Forster & Livingston (25) and confirmed the latters' finding that the older Prins value for the yield was too low. Both of the newer sets of observations show a yield for chlorophyll *a* independent of solvent (ethyl ether, acetone, methyl alcohol, benzene, cyclohexanol, pyridine) and a yield for the *b* component just half as great as for *a* in ether and about a quarter as great in methyl alcohol. The newer yields reported from Urbana, however, are about 40 per cent higher than those from the Minnesota group and this difference is consistent for all the measurements. Thus the yield for *a* in ether is 0.33 or 0.24 and for *b* in ether 0.16 or 0.11, as reported by these two laboratories, respectively. The discrepancy, not too important for photochemical consequences, may be due, as Latimer suggested, to systematic errors in the calibration of the thermopile or in the positioning of the elements within the sphere.

A very spectacular recent study was the direct determination of the lifetime of fluorescence by Brody (26). Previous estimates were based either on a theoretical interpretation of the integrated area under the absorption spectrum curve or on a simplified model of the effect of viscosity on the mobility of an excited chlorophyll molecule as manifested experimentally in the depolarization of fluorescence. In Brody's newest method, fluoresence is excited by a very short light pulse from a hydrogen discharge tube. The decay of the resultant fluorescence is translated into an electrical signal and impressed directly on the plates of an oscilloscope. The very fast time resolution, of the order of 10^{-9} sec., was made possible by the use of specially designed flash tubes, high pulsed voltages to operate the photomultiplier tubes, very careful general electrical design to eliminate needless circuit contributions to the response time, and a detailed statistical analysis of the oscilloscope trace. The observed lifetimes, in units of 10^{-9} sec., were as follows: chlorophyll *a* in benzene, 7.8; in ethyl ether, 5.1; in methanol, 6.9; chlorophyll *b* in benzene, 6.3; in ethyl ether, 3.9; in methanol, 5.9. These values were obtained in solutions so dilute that self-quenching was negligible and the lifetime was independent of concentration. These values, when divided by

Latimer's values for the fluorescent yield, gave figures for the natural lifetime (the hypothetical mean life if the excitation energy could be lost only by fluorescence and all other means of dissipation were excluded) in remarkable agreement with values predicted by the analysis of the absorption curve suggested by Förster (27).

French has recently reviewed the cumulative work at his laboratory on the precise determination of the fluorescence spectra of the photosynthetic pigments, both *in vitro* and *in vivo* (28). He has employed ingenious techniques for the elimination of reabsorption of fluorescent light and for the analysis of multicomponent fluorescence curves. In connection with studies on chlorophyll orientation in grana, Arnold & Meek (29) and Goedheer (30) measured the polarization of the fluorescence in very viscous chlorophyll solutions. Both laboratories reported a disappearance of polarization at 580 mμ, indicating that the small absorption peak at this wavelength represents a different electronic transition than the main 660 peak. Goedheer also reported a splitting of the Soret peak into two components representing electronic transitions perpendicular to each other, both within the plane of the porphyrin framework. These observations are in agreement with current theories of electronic spectra, but the details have some points of variance with previously published work of Stupp & Kuhn (31). Rabinowitch suggested a possible contamination of the preparation with pheophytin to account for Stupp and Kuhn's results (4).

Sensitized reactions.—The kinetics of several chlorophyll sensitized oxidation-reduction reactions have been studied in such great detail that it is possible to draw definite inferences as to the mechanism of sensitization. Livingston & Owens reinvestigated Gaffron's actinometer reaction, the autoxidation of allyl thiourea (32). Previous measurements on the extreme sensitivity of chlorophyll's metastable state to traces of oxygen suggested that the first photochemical step is the formation of a complex between oxygen and chlorophyll's metastable state. In the absence of a reducing substrate this complex quickly dissociates with dissipation of the excitation energy. In the presence of allyl thiourea, the oxidation-reduction occurs with the regeneration of chlorophyll. This last step is the slow step of the reaction sequence, and at low reductant concentrations this step competes unfavorably with dissociation of the complex. The dissociation occurs everytime a molecule of the complex diffuses to the neighborhood of a normal chlorophyll molecule. Although this mechanism had been proposed before, the newest paper of Livingston & Owens shows that actual rate constants for the individual steps of the sequence can be computed which are consistent with data from independent experiments, including fluorescence yields and lifetime, metastable state decay kinetics, fluorescence quenching, and quantum yield of the oxidation. Throughout this whole analysis (33), the striking high reactivity of oxygen for excited chlorophyll, whether in the fluorescent or metastable state is apparent, the attachment of oxygen occurring at almost every encounter. The nature of the oxygen complex is not definitely known. By

analogy to the photochemical synthesis of ascaridol by oxidation of α-terpinene, it has been suggested that the oxygen adds as a bridge across two points of the chlorophyll conjugation network [Schenck (34)]. This suggestion for chlorophyll must still be regarded as speculative. One of the mysteries of photosynthesis is the protection of the photochemical apparatus in the plant from this type of oxygen-induced dissipation. This is the basis of Franck's view that the immediate reactant with chlorophyll in photosynthesis cannot be a biocatalyst, but must be a substrate present in large enough concentrations to compete with oxygen at the chlorophyll surface (35).

By surveying the data for a wide variety of chlorophyll sensitized oxidation-reduction reactions, Livingston & Pariser (36, 37) were forced to conclude that the rapid attachment of oxidant to the photoexcited sensitizer (in its metastable state) cannot be a general mechanism. In the sensitized reduction of azo dyes by a variety of reductants, it was found that the fall off of the quantum yield in limiting amounts of dye did not occur at the same dye concentration for all reductants. If the dye had attached itself primarily to excited chlorophyll, the concentration effect of dye would have depended only on the competition between the attachment reaction and the normal decay of excitation, neither process involving the reducing agent. The results can be explained by postulating that the dye reacts with an excited chlorophyll—reductant complex, again formed from the metastable state. In this case, the fall off of yield depends on the competition of the dye for this complex which may well have a rate dependent on the specific nature of the reductant in the complex. In the cases studied most exhaustively, the quantum yield approached a maximum value of 0.5 per dye molecule reduced. This indicates that the two-electron transfer proceeds by successive one electron (or preferably one hydrogen atom) steps.

These latter conclusions are, of course, in keeping with the current interpretations of the Krasnovskii reaction. This is the reversible photobleaching of chlorophyll by reductants in more or less basic solutions. Since the reduced chlorophyll, characterized by its absorption spectrum, can be re-oxidized by subsequently added oxidants in the dark, the photobleaching can be regarded as the first set of steps in a complete sensitized oxidation-reduction. Evstigneev & Gavrilova found that at least two spectroscopically distinguishable reduced forms of chlorophyll must be present in the photo-bleached state (38). They suggested that one of these may be a semiquinone resulting from the transfer of one hydrogen atom and the other may be its conjugate base. A study of the infrared spectrum of the bleached form might provide information about the site and nature of the reduction, particularly if it occurs near ring V. The large Russian literature on this reaction, unavailable in most American laboratories, has been summarized in great detail by Rabinowitch in the last volume of his monograph (4).

Linschitz carried out a Krasnovskii type reaction in a low temperature rigid glassy medium where molecules are not free to move (16). With phenyl-

hydrazine as reductant, he observed a photobleaching of chlorophyll *a*, with appearance of strong peaks at 525 and 400 mμ but no absorption in the red or far red, in accordance with the spectrum reported by Evstigneev & Gavrilova (38). Linschitz was unable to observe paramagnetic resonance absorption in the bleached material and was thus unable to demonstrate experimentally the free radical nature of the intermediate. As pointed out above, however, a negative result in this type of experiment is not conclusive. A direct determination of the magnetic susceptibility by some kind of magnetic balance would be the most conclusive type of test. The recent studies of Krasnovskii & Umrikhima (39), reminiscent of those of Uri (40), showing the initiation of polymerization of methyl methacrylate by photochemical systems containing chlorophyll, pyridine, and reducing agent, cannot be considered conclusive. Although free radicals are known to catalyze polymerization reactions of this type, the electronically excited triplet states might also possess this property. The appearance of the Krasnovskii intermediate under conditions where molecules are not free to move places certain restrictions on the nature of the reaction. If the reaction requires interaction of chlorophyll with the reductant, a complex of these two species might have to be formed before irradiation. Such a complex would have to possess the same absorption spectrum as chlorophyll. The photochemical reaction might involve an intramolecular shift of a hydrogen atom from the reductant to the chlorophyll within the complex. Such a shift, if a small one, need not require a fluid medium. These experiments are usually carried out with over a thousand-fold excess of reductant, so that the preillumination complex need not be too stable to make such a mechanism plausible. Such a possibility had been pointed out previously by Livingston & Pariser (37) in connection with a generalized mechanism for sensitized oxidation-reduction reactions.

Although the above oxidation-reduction reactions are all presumed to proceed by way of the long-lived metastable excited state, the possibility of direct reaction with the fluorescent state in some cases should not be dismissed. Fujimori discussed this possibility in the case of the chlorophyll sensitized reduction of a tetrazolium salt by hydrazine hydrate (41). Although the evidence in this particular case is not convincing, we will see in connection with photosynthetic green cells (see below) that such a direct coupling of the fluorescent state with the reaction substrate is not only possible but likely in some instances. One advantage of this mechanism is the availability of about 25 per cent more energy in the fluorescent state than in the metastable state. The price that must be paid, to take advantage of this benefit, is a very high concentration of substrate, at least 0.01 *M* for reactions in solution, to insure a high probability of encounter with the fluorescent state during its short life time, or alternatively the formation of a preillumination dye-substrate complex.

The photobleaching of dyes by reducing agents is not limited to chlorophyll. Oster and co-workers have shown, for example, that the photoreduction of methylene blue and thionine by ascorbic acid or phenylhydrazine

XUM

proceeds by way of the dye's metastable state, as evidenced by the lack of fluorescence quenching by these substrates (42). The reaction requires the diffusion of the two reactants toward each other as is made clear by the strong viscosity dependence of the quantum yield. The photoreduction of eosin with allylthiourea follows the same general pattern (43), and reaches the limiting quantum yield of 0.1. Here, as in the other cases, the metastable state of the dye decays spontaneously or by collision with another dye molecule unless the substrate approaches quickly. In the above cases, the bleached form may be at least partially reconverted to the original dye by a subsequent reaction with a suitable oxidizing agent. One feature of Oster's work that is very suggestive for biological systems is the strong change in the course of these photoreductions when the dye is adsorbed on a solid surface (44). Acriflavine, for example, cannot be photoreduced by ascorbic acid unless adsorbed on the surface of an acidic polymer. One known effect of adsorption of such dyes is the increased stability of the metastable state.

In addition to the authentic photo-oxygenation of chlorophyll in the presence of molecular oxygen (see above), there has been speculation from time to time about reversible oxidations not involving molecular oxygen. Ferric and ceric ions have been known for some time to promote photobleaching which is reversible on addition of reducing agents [Rabinowitch & Weiss (45)]. Linschitz & Rennert's original note on the freezing in of a photo-bleached form of chlorophyll formed in rigid medium in the presence of quinone suggested a dye oxidation and a quinone reduction (46). Such an intermediate had often been looked for as an alternative to reduced chlorophyll as the agency for oxidation—reduction catalysis. Although a primary dehydrogenation of chlorophyll followed by a subsequent hydrogen transfer from the reductant was ruled out by the tracer experiments of Weigl & Livingston in the azo dye-ascorbic acid system (47), such a mechanism has not been ruled out for other cases. Linschitz's recent reinterpretation of his experiments with quinone discards the oxidation hypothesis and proposes instead that the spectral changes, with new absorption peaks at 705 and 530 mμ, correspond to ionization (or enolization) of chlorophyll *a* at C-9 (16). This conclusion was based on a comparison with the newest determinations of the spectrum of chlorophyll ionized at C-9, such as the intermediate in the Molisch phase test [chlorophyll plus alkoxide in pyridine (48)] or in the low temperature reaction of chlorophyll with amines (49). Supporting evidence was cited in the enhancement of the quinone reaction by bases and in the absence of a reaction with allomerized chlorophyll or synthetic tetraphenylchlorins (without ring V).

Photochemistry *In Vivo*

Spectroscopy.—There has been a recent flurry of activity, both experimental and theoretical, in attempting to define two kinds of chlorophyll *a* in the plant, spectroscopically distinguishable. Krasnovskii & Kosobutskaya drew such a conclusion on the basis of some data on the biosynthesis of

chlorophyll (50). The evidence cited is that the first chlorophyll formed in the greening of etiolated tissues has an absorption maximum at 670 mμ, in contrast to the 678 mμ peak ultimately attained by a fully greened specimen. Smith has also reported the lack of photosynthetic activity during the first few minutes after formation of chlorophyll (51). Krasnovskii and coworkers have concluded on the basis of this type of evidence that active and inactive forms of chlorophyll exist in the mature green plant, although they have not been resolved spectroscopically. The inactive form, in their view, is fluorescent and serves as an energy collector for use, after resonance transfer, by the nonfluorescent active form. Rabinowitch, in reviewing this topic in the last volume of his monograph, discussed the differences of view of Smith and Krasnovskii as to which of the two forms is photochemically active, and pointed out that the evidence for the co-existence of the two forms in the fully greened leaf is very weak indeed (4). The forms recognized in the greening process may represent merely stages in the development of the chlorophyll-lipoprotein layered structures in the grana. Brody, in attempting to interpret his *in vivo* fluorescence data (see below), has invoked the possibility of the co-existence of two kinds of chlorophyll in *Chlorella*, the nonfluorescent type constituting three-fourths of the total (26). As independent evidence of this possibility, he recalled the small minimum at 660 mμ in the plot of quantum efficiency versus wavelength, as reported by Emerson & Lewis (52). This might now be interpreted as arising from the inefficient transfer of energy from the inactive form, absorbing at 660 to 670, to the active form, having its maximum at 678 mμ.

Several recent methods have been reported that may be useful for elucidating details of the pigment absorption spectra of plant cells. Latimer & Rabinowitch studied the spectral dependence of light scattering by *Chlorella* (53). Superimposed on the nonselective scattering, which increases regularly with decreasing wavelength, they found maxima and minima near the absorption peaks of the main pigment components. These are a consequence of rapid changes of the refractive index near absorption peaks, and manifest themselves as scattering maxima on the long wavelength side of absorption maxima and scattering minima on the short wavelength side. Scattering maxima were observed in *Chlorella* at 690, 510, and 460 mμ; scattering minima at 650, 470, and 430; absorption maxima at 680 and 440 attributable to chlorophyll and a carotenoid shoulder at 465. The 510 scattering peak was extremely sharp and provides a much more striking record of the carotenoids than does the absorption spectrum. Goedheer (54) and Menke & Menke (55) have made observations of selectivity in the birefringence of leaf chloroplasts, arising from the same fundamental source as the scattering phenomena. These latter investigations were undertaken primarily to study orientation in the chloroplast, a topic reviewed elsewhere in this volume by Clendenning (6). Virgin has discussed and utilized the advantages of infiltration of leaves by water prior to fluorescence measurements in order to reduce scattering caused by air pockets (56). Duysens has treated the flat-

XUM

tening of the absorption spectrum of cell suspensions attributable to the mutual shading of pigment molecules in the highly colored plastids. He has provided experimental procedures for converting raw absorption data to the corrected curve that would be obtained if the pigment molecules in a given volume of suspension were uniformly distributed throughout the suspension (57).

French and co-workers have continued to develop the technique of derivative spectroscopy, which measures directly the derivative of the transmission with respect to wavelength (58). Although the techniques have very little similarity, the direct recording of a derivative spectrum has been used for some time in microwave spectroscopy. The derivative curve is sharper than the direct transmission curve, and is particularly useful for resolving a minor component that would appear only as a shoulder on an ordinary absorption curve.

Steady state fluorescence.—Brugger (59) and Latimer *et al.* (24) have reported new measurements on the dependence of steady state fluorescence in *Chlorella* upon light intensity. Both investigations showed a noticeable drop in the fluorescence yield at intensities below compensation. Brugger estimated a two-fold increase in the slope of the fluorescence versus incident intensity curve in the half-saturation range over that below the compensation point. Latimer, reporting his data as fluorescent yield (the ratio of fluorescent quanta to absorbed quanta) found a 40 per cent increase at ten times the compensation intensity over the yield at 1 per cent of the compensation. Brugger's observation that the marked decline of fluorescent yield at low light does not occur anaerobically suggests the connection of the decline with respiratory processes. Franck, in adapting his explanation of the Kok effect to this new phenomenon, suggests that the fluorescent state can be used directly for the photochemical reduction of energy-rich intermediates of respiration, as opposed to the normal course of photosensitization at higher intensities by way of the metastable state (60). The fluorescence is quenched at low light because the respiratory intermediate is present in high enough concentration and has a high enough energy content to abstract a hydrogen atom from chlorophyll in its fluorescent state. Franck suggested 1,3-diphosphoglycerate as such an intermediate.

Latimer's absolute determinations of the quantum yield of fluorescence in *Chlorella* were in the range of 2.5 to 3.5 per cent, depending on the incident intensity. These are about one-tenth of his reported yield of chlorophyll *a* fluorescence in organic solvents. It has always been known that green plants fluoresce more weakly than chlorophyll solutions and this difference has been ascribed to the more efficient migration of energy into the metastable state in the case of the chloroplast. Simple theory would require that the lifetime of the fluorescent state be lowered by this same factor of 10. Brody's direct measurement on *Chlorella*, however, showed that the lifetime of fluorescence is lowered by just a factor of three (26). To explain this anomaly, he advanced several possible reasons. (*a*) Some chlorophyll in the

plant might be completely nonfluorescent, and would make no contribution to the observed lifetime kinetics of the other fraction. This was discussed above in connection with the absorption spectroscopy of newly-greened leaves. (*b*) The lifetime measurements were made with repeated pulses of high intensity light flashes, the instantaneous intensity of which was 2500 times higher than the steady light levels of Latimer's experiments. Brody himself rejected this hypothesis by considering that in each flash only about one chlorophyll molecule in a million was excited. This low average degree of excitation rules out any of the current theories which account for the slight rise of fluorescence above saturation intensities as a manifestation of a more or less stoichiometric change in the complexing of chlorophyll to reaction intermediates or to natural protective narcotics. (*c*) Brody's algae might actually be suffering from too little light, on a time average basis, and may never have come out of the induction period, which customarily shows higher fluorescence yields in the beginning of a light period. All of these attempted explanations are based on the inherent assumption that the basic pathways of de-excitation of the fluorescent state are the same in the chloroplast as in ether solution. Although the similarity in shape of the red absorption curves in the two cases suggests that the natural lifetime should be the same, this conclusion is essentially one of faith rather than proof. The comparison of the flash bleaching experiments between chlorophyll in solution and in the plastid raises some doubt about the basic similarity of the de-excitation processes in the two cases (see below).

An interesting result of Brody's work is a new type of experimental confirmation of energy transfer among the various pigments of the plastid (26). An analysis of the experiments with chlorophyll in solution or with algae illuminated in the absorption band of chlorophyll *a* shows that fluorescence decays exponentially starting with the instant of excitation. If the red alga, *Porphyridium*, is illuminated in the absorption band of phycoerythrin, however, the observed chlorophyll fluorescence begins to decay exponentially only after a time lag of 5×10^{-10} seconds with respect to excitation. This holdup time, if interpreted as the mean time required for energy transfer from phycoerythrin to chlorophyll by way of phycocyanin, is remarkably consistent with the rates of energy transfer calculated from the observed efficiencies of sensitized fluorescence in this complicated pigment system (61). Another independent experimental confirmation of energy transfer was reported by Arnold & Meek on the basis of their study of the depolarization of fluorescence in *Chlorella* suspensions (29).

Transient spectroscopy.—Since evidence began to accumulate for the participation of the metastable state in chlorophyll's *in vitro* photochemical reactions, the acceptance of a similar role for the metastable state in photosynthesis has been tempting. The relatively low fluorescence yield in green plants, even at intensities far above the saturation level, has been interpreted as evidence that fluorescence is quenched by a very efficient transfer of energy into a metastable state rather than by direct photochemical reac-

XUM

tions (35). The direct experimental confirmation of this hypothesis has turned out to be very difficult indeed. A long wavelength phosphorescence, such as has been observed for many dyes *in vitro* including chlorophyll *b* (see above), has never been found in green plant cells or in chloroplasts. The red afterglow of the same spectral distribution as plant fluorescence, discovered by Strehler & Arnold, seems to be much too complicated a phenomenon to be interpreted as the usual metastable state phosphorescence (62). The dependence of the afterglow on temperature, on inhibitors, and on incident light intensity, and especially the time course of the luminescence, all suggest that some kind of recombination, chemical or electronic, is involved. Arnold's observation (63) of a very slowly decaying tail up to many minutes in the dark seems to make implausible the earlier interpretation that short-lived chemical intermediates are wholly responsible for the effect. The wide range of stabilities of the intermediates as reflected in the broad distribution of apparent rate constants of recombination is suggestive of a pseudo-crystalline structure in the grana which can trap fragments of ionization or dissociation in lattice imperfections. The present writer feels that the time is not ripe for a comprehensive review of the nature and significance of the afterglow because of the importance of new work in this area, not yet published, by Arnold and by Franck. Perhaps next year's volume in this series would be a more appropriate place for further discussion of this phenomenon.

The other possible experimental approach to the metastable state in plants is the spectroscopic study of steady state or flash bleaching, analogous to the methods employed for chlorophyll solutions. Both methods have indeed been tried. Duysens (3) reviewed the experimental results on the steady state reversible bleaching of green cells in the light. These small changes in the absorption spectrum were interpreted as manifestations of cytochrome oxidations and pyridine nucleotide reductions in the light. The relationship of these changes to the primary photochemical process was not certain, and the correlation of these changes with changes in the chlorophyll molecule itself seemed to be ruled out by the absence of changes at the wavelengths of chlorophyll's maximum absorption. Since Duysen's review appeared, Coleman, Holt & Rabinowitch have reinvestigated the small photo-induced changes in *Chlorella's* absorption spectrum (64). Contrary to the results of previous workers, they found a decrease in absorption at 680 mμ, the region of maximum chlorophyll absorption by green plants. This datum seemed to indicate that chlorophyll may indeed undergo a reversible modification in the light as part of its photocatalytic role. Whether this decrease at 680 mμ was associated with the same pigment responsible for the previously known decrease at 475 mμ and increase at 515 mμ was uncertain. If chlorophyll had been converted to its metastable state, the 475 mμ bleaching is in the wrong direction. If the modified form were the Krasnovskii reduced form, the 515 mμ increase would be explicable but the 475 mμ effect would still have to be attributed to something else. In any event, the maxi-

mum change in the optical density near the 680 mμ region was about 0.25 per cent. Several experimental pitfalls associated with such small fractional changes have already been pointed out.

One problem in all differential spectroscopy of scattering systems is the complication of the scattering anomalies occurring near absorption peaks (53). Latimer has pointed out that small changes of absorption, in the parts per thousand range, not measured in an integrating sphere, may be distorted appreciably as a result of selective scattering (65). This difficulty applies particularly to attempts at correlating such difference spectra with compounds of known absorption. For another thing, the red peak overlaps the region of chlorophyll fluorescence. In the cross-illumination technique, the actinic light is provided by a strong lamp shining through the sample at right angles to the very weak spectroscopic beam. If the detector responding to slight changes in absorption of the spectroscopic beam is provided with appropriate filters, light scattered by the sample can be eliminated. But if the detector is to respond to a wavelength in the red, the red fluorescence resulting from the strong actinic light cannot be excluded. Various experimenters have allowed for this fluorescence interference in several ways. Some have assumed that the fluorescence signal is independent of the presence of a spectroscopic beam and have used the signal from the actinic light alone as a blank. Coleman *et al.* (64) resorted to a modulation technique to eliminate the fluorescence. They chopped the spectroscopic beam at a fixed frequency and amplified the detector's response signal with an electronically tuned circuit that discriminated against all frequencies except that of the modulation. Thus, fluorescence arising from the unmodulated steady actinic source would be eliminated during the amplification process.

Strehler pointed out that an assumption is inherent in this procedure, that the weak fluorescence due to the modulated light beam is independent of the presence of the actinic light (66). This assumption is probably not valid, because it is well known that the fluorescence yield increases with increasing intensity of illumination (24, 59). As a result, the detector will see more fluorescent light from the modulated beam when the actinic light is on, and this will appear to result from a decreased absorption. This question might be settled by performing experiments with the modulated beam monochromatized both before and after passage through the cell suspension and thus reducing the proportion of fluorescent light reaching the detector. Strehler & Lynch performed independent measurements of the reversible optical absorption, confirming the previously reported changes in the blue and green and reporting no changes at 680 mμ or above (67). Their technique consisted of a flow method in which the differential absorption was measured across two points in the flow path, one in the light and one in the dark. They found some of the same phenomena as earlier workers, but found in addition profound influences of the intensity and duration of illumination on the magnitude, and even sign, of the difference spectrum.

Witt has reported further details of his measurements of difference spec-

XUM

tra following flash illumination (68). The increased absorption at 515 mμ and the corresponding decrease at 475 mμ occur in less than 3×10^{-5} sec. for flashes lasting about a millisecond or less. The normal spectrum is restored by a temperature-dependent dark process having a half time of about 0.01 sec. at 25°C. For flashes larger than a millisecond, the substance responsible for the transient spectrum is built up in a second slower stage to a level about two or three times that reached in the rapid initial stage. The rapidity of the first stage of the absorption change suggests that a direct photo-process is involved, and the temptation is great to ascribe the change to a chemical or at least electronic modification of the chlorophyll. The absence of evidence for the lowering of the main chlorophyll absorption peaks, however, casts doubt on this interpretation, as discussed in the preceding paragraphs. The strong similarity in the kinetics of the absorption changes and of chlorophyll fluorescence, the similarity of the saturation curves of the colored transient development and photosynthesis, and the similarity in susceptibility to narcotics all are suggestive of a close relationship, if not identity, between this new substance and an early photo-product in the photosynthetic sequence. The suggestion has been made from time to time that chlorophyll *a* is not the point of direct contact between optical excitation and the photosynthetic substrates. If a pigment is present in concentrations too small to be detected in normal spectroscopy, its excited state might show up plainly in the differential spectroscopy of a flash experiment. This pigment would have to absorb farther into the infrared than chlorophyll so as to act as an efficient energy sink for resonance transfer.

The search for the metastable state in green plants was carried out also by Rosenberg, Takashima & Lumry, using the flash technique (69). They used short flashes containing enough energy to excite all the chlorophyll molecules in their preparations several times, just as in the flash spectoscopy of chlorophyll solutions. They were unable, however, to detect any reversible spectroscopic change within their estimated 5 per cent limit of detection. From these negative results they concluded that (*a*) either the metastable state is not a pathway in the photosynthetic chain for most of the chlorophyll molecules, or (*b*) the main reaction sequence goes through the metastable state in less than 10 μsec. The first of these possibilities is equivalent to one of the modifications of the photosynthetic unit, to be discussed further below, in which several hundred chlorophyll molecules pool their absorbed energy for a single reaction site; the persistence of excitation for a time long enough to be observed spectroscopically occurs only at the special reaction site. The second possibility, the very rapid occurrence of the primary photochemical step, suggests that the direct energy acceptor for chlorophyll is a nondiffusible constituent of the chloroplast, perhaps the protein attached to the chlorophyll. This requirement is imposed by the absence of an observable spectroscopic change in well washed chloroplast preparations thoroughly freed of dissolved oxygen. These experiments are not surprising in view of Witt's finding that with much weaker light flashes a saturation of

spectroscopic changes sets in at a level corresponding to a few tenths of a per cent of the optical density based on all the chlorophyll in the plant. The consistency of these results, however, does not simplify the problem of explaining the apparent widely different sequence of events following chlorophyll excitation *in vitro* and *in vivo*.

Flashing light experiments.—Kok & Businger reported new results on flashing light experiments, which they interpret as evidence for the photosynthetic unit (70). Confirming the older results [see review by Lumry *et al.* (1)] on the temperature independence of the maximum yield from very short flashes (less than 10^{-4} sec.) and on the dark time for the reaction responsible for saturation in steady light (.02 sec.) they presented quantitative evidence for a new dark period shorter than the classical one. This is interpreted as the time required for regeneration of a light absorber or of a substance that receives its energy directly from the light absorber, such as by resonance transfer. This new dark time, in the millisecond range, accounts for the dependence of the flash saturation yield on the length of the flash when the latter is from 10^{-4} to 10^{-2} sec. An additional constant coming out of these studies is the concentration of the primary photochemical substrate. Summarizing these results, the authors would say that: (*a*) a photosynthetic unit exists consisting of about 200 chlorophyll molecules that all funnel their energy to a common site; (*b*) there are about two or three times as many primary photochemical substrate (or enzyme) molecules as photosynthetic units; (*c*) a primary substrate molecule reacts with the reactive site of the unit slowly, so that a few milliseconds are required to regenerate the reactive site for subsequent use; (*d*) the primary substrate reacts with the next member of the chain and reactivates itself in about a fiftieth of a second. It should be pointed out that this is not a unique interpretation of the data. The results can be interpreted also by postulating a sequence of enzymatic steps, each with its own characteristic reaction time and its own saturation level regulated by concentrations. Additional experiments were reported on photo-inactivation of photosynthesis by strong light. That the quantum efficiency at low light was impaired at the same time as the maximum flash yield for brief flashes was interpreted by the authors on the basis that an entire photosynthetic unit is damaged by destruction of any of its parts. An alternate explanation could be advanced, however, in terms of the nonselectivity of photochemical destructions, as for the well documented cases of ultraviolet and narcotic inactivation. In all these cases the inactivation applies at both low light and high light. All of these inactivating agents are nonspecific in nature and could act simultaneously on the chlorophyll and on any enzyme whose limitation is responsible for saturation.

Two-quantum picture.—The most comprehensive self-consistent picture of the photochemical aspects of photosynthesis is attributable to Franck. In order to interpret some of the newer theoretical and experimental advances, Franck has made a number of modifications of his earlier views. Although the complete details of his newest theory are not yet available

XUM

at the time of this writing, some of the novel features have already been presented in preliminary reports (35, 60). The major point of departure from earlier schemes is the proposal that two quanta of excitation must reach the same pigment molecule before the basic photochemical act of photosynthesis can occur. This proposal is unique among known photochemical mechanisms. It was offered because photosynthesis offers problems that seem to be unique in their complexity. One of the particular difficulties that this solution is designed to meet is the need to accumulate enough energy to break an O—H bond of water. Photosynthesis is one of the few known examples of photochemical reactions proceeding with high efficiency in which a net storage of chemical energy occurs, and the amount of energy stored per quantum is far greater than in any other of these few. The energy-requiring reaction is the transfer of a hydrogen atom to the primary oxidant. Uri has recently reviewed the urgency of this problem in terms of known bond strengths in water, semiquinone radicals, and organic peroxides (71). Duysens reviewed various recent proposals involving multiple oxidation-reduction reactions over small standard potential differences, followed by chemical or energetic dismutations leading to the formation of a strong reductant and a peroxide (3).

Franck's newest proposal would avoid some necessity for a chemical bucket brigade up the energy ladder by accumulating the energy of two quanta before chemistry occurs. With the energy of two quanta, it is quite feasible energetically to form simultaneously a monohydro-reduced radical leading to carbohydrates and a peroxide radical. Coupled with this picture is a detailed model for the collection of two quanta at the same place. The first quantum would be used to excite some chlorophyll molecule to its metastable state. This long-lived metastable state, by virtue of its biradical character, would attract the reducible substrate. If the metastable state has an absorption maximum at 700 mμ or slightly higher, as indicated in Linschitz' experiments *in vitro* (16), then the probability of resonance transfer from the fluorescent state of any neighboring chlorophyll molecule to it would be greater than to a normal chlorophyll, and hence would be a very efficient process. The result would be a molecule in a more highly excited metastable state. Franck has, in addition, proposed specifically that the primary substrate is phosphoglyceric acid (PGA) in keeping with the requirement he has stressed so often that the direct oxidant must be present in large amounts to preserve high efficiency of contact and to prevent the access of oxygen with its accompanying dissipative effect on the excited dye. He believes that the oxidant is attached near C-10 of the chlorophyll and that the labile hydrogen at this position is the one transferred. An enzyme involved in oxygen liberation is attached near C-9 which is previously assumed to have been hydrated. The photochemical reaction consists of the simultaneous transfer of a hydrogen atom to PGA and of an —OH to the oxygen liberating system. The half-reduced PGA might undergo a dismutation with another of its kind to form one PGA and one triose phosphate. The —OH-

containing enzyme is destined to furnish the peroxide for ultimate oxygen liberation. The chlorophyll is then left in its enol form, and must ketonize and rehydrate before it is ready to function again. The dark times of the flashing light experiments might be related to the desorption of the semiquinone or of the peroxide or to the tautomerism of the chlorophyll itself.

One attractive feature of this model is the relationship of fluorescence to photochemical activity. Although the principal reason for quenching of chlorophyll fluorescence *in vitro* is the intramolecular conversion to the metastable state, an additional important quenching process *in vivo* seems to be intermolecular resonance transfer to other pigment molecules in the granum. The resonance transfer is favored by the high local concentration of chlorophyll, by the partial orientation of the chlorophyll molecules, and by the existence of an energy sink, which in this case is that molecule previously excited to its lowest metastable state. Brody's directly measured value of the fluorescent lifetime in *Chlorella*, 1.6×10^{-9} sec., allows at least 1500 transfers of excitation from one chlorophyll molecule to another before the excitation is dissipated by nonphotochemical processes (26). This determination removes one of the difficulties previously ascribed to the transfer hypothesis by Franck on the basis of a much lower estimate of the lifetime (72).

Fluorescence is still possible while the excitation is being transferred from the fluorescent state of one molecule to that of another; but fluorescence becomes impossible as soon as the excitation leaves the fluorescent state and reaches the metastable state of the energy sink. Thus the existence of the sink acts to lower the fluorescence. The observed rise of the fluorescence yield with increasing intensity of illumination, particularly above saturation, is attributable to the blocking of the sink positions by enzymatic limitations. On this view, one might predict that fluorescence would be an increasingly important dissipation process at very high intensities such as those used by Rosenberg *et al.* in the flash experiments (69). This hypothesis could be confirmed if the fluorescence yield were found to increase appreciably in strong flashes. Under such conditions, there should be also an increased yield of metastable state population. The failure to observe it might mean that the energy of the metastable state is quenched dissipatively with high probability in the granum. The high efficiency of photosynthesis at low light would then require that certain molecules in their metastable state, those serving as energy sinks for collection of a second quantum of excitation, must be protected against quenching in some way. Such a favored situation of one chlorophyll molecule out of many is indeed possible on the basis of geometry. Molecules exposed to an aqueous phase, for example, might be complexed quite differently than molecules imbedded in a lipide phase. If certain chlorophyll molecules were indeed located in favored positions for participation in chemical sensitization, an additional requirement would have to be added that the favored molecule have an absorption further to the red than the bulk of the pigment molecules so that energy could migrate preferentially to the site where it could be used for chemical action.

XUM

To account for the observed quenching of fluorescence by PGA and by Hill oxidants like quinone, Franck has postulated an alternative photochemical pathway involving a hydrogen transfer to the oxidant directly by the fluorescent state. For energetic reasons, the —OH at C-9 cannot be split off until a second quantum arrives. This must be a minor process compared to the concerted one involving the metastable state; otherwise, the fluorescence would rise much more than it does under conditions of enzymatic limitations.

The minor process, direct photochemistry by the fluorescent state, can become the important pathway at low intensities. As a matter of fact, a two-quantum process becomes improbable at low intensities because a given molecule cannot live long enough in its metastable state to await the arrival of a second quantum [Clendenning (73)]. Assuming a two-dimensional packing of chlorophyll in the sites of a quasi-lattice, we can calculate that a quantum destined for effective photochemistry will be absorbed within about 50 lattice sites of any designated position every millisecond at saturating intensities. This is not too large a distance for resonance transfer, and a millisecond is not an unreasonable life time to expect for the metastable state. At very weak intensity, either the sink would have to live longer to collect a quantum from the same area or it would have to be eligible for energy initially absorbed over a wider area. Partly to avoid this difficulty and partly to interpret the effect of respiratory intermediates on the photosynthetic balance at low light, Franck postulated that energy-rich compounds can substitute for PGA as the primary oxidant below the compensation point. Specifically he spoke of 1,3-diphosphoglyceric acid as a substitute oxidant. The added acyl phosphate bond energy in this compound makes a one-quantum reduction energetically feasible.

One other and separate point made by Franck in his recent papers deserves mention here (60). This concerns the possibility that photosynthesis may involve electron transfers in the sense of a solar battery. Such suggestions have frequently been made. Franck is very pessimistic about such possibilities for very fundamental reasons. Whenever a charged particle, such as an ion or electron, is created in an aqueous medium, the particle will tend to orient water molecules about it by ion-dipole attraction. This orientation, however, requires time of the order of 10^{-10} sec. because of the moment of inertia of a water molecule. If a charged particle is formed under conditions where this orientation cannot occur, then the energy cost of the ionization process cannot be compensated by the hydration energy. In the absorption of light by molecules, an electron is excited in a time very short compared to the rotational orientation time of water. Thus, an ionization process must result, in the first instant, in a poorly hydrated ion. The energy requirement for such a process makes this type of mechanism out of the question for visible light and becomes just barely possible in the medium ultraviolet. Therefore we should anticipate that reaction intermediates in the rapid reactions of photochemistry are not newly created ions but free radicals resulting from bond dissociation and atom transfer.

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BIOCHEMISTRY OF CHLOROPLASTS IN RELATION TO THE HILL REACTION[1,2]

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Introduction

The mechanism of photosynthesis has been greatly clarified by recently completed investigations, which are discussed in a number of recent reviews (1 to 12). This progress has narrowed the gap in information essentially to the photochemical driving mechanism. The Hill reaction lacks the intricate synthetic reactions of photosynthesis, but it transforms light into chemical energy and retains other features of the photochemical phase. In photosynthesis, active hydrogen obtained photochemically from water is consumed in PN reduction, and in the coupled formation of ATP, these being the first water-soluble compounds in which photochemical energy is known to appear (8, 9, 11); in the Hill reaction, the active hydrogen is shunted by an artificial oxidant. Photosynthesis in plants is energetically superior to bacterial photosynthesis, the latter proceeding only in the presence of energy-rich hydrogen donors. The site of the plant's superiority in this respect is closely associated with the photochemical production of oxygen from water, which is retained in the Hill reaction. The use of isolated chloroplasts and of artificial oxidants introduces numerous problems, but the Hill reaction allows the photochemical driving mechanism of photosynthesis to be analyzed under simplified conditions.

Chloroplast structure, growth and inheritance has been reviewed recently by Weier & Stocking (13), Rhoades (14), Granick (15) and Rabinowitch (1). Advances in the general field of this paper have been discussed in reviews of this series (3, 8, 11, 18, 20, 21, 22) and elsewhere (1, 2, 5, 8, 10, 11, 12, 16, 17, 19). This review is concerned mainly with chloroplast isolation problems and the mechanism of water photolysis.

General Considerations

Photosynthesis and the Hill reaction appear to be invariably associated with chlorophyll bound in layered lipoprotein structures. These laminated structures are borne either in chloroplasts or in "free" grana resembling

[1] The survey of the literature pertaining to this review was concluded in September, 1956.

[2] The following abbreviations will be used: AMP, adenosinemonophosphate; ATP, adenosinetriphosphate; DNP, dinitrophenol; PN, pyridine nucleotide; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; R.C.F., relative centrifugal force.

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those found in chloroplasts (1, 15). Blue-green algae and photosynthetic bacteria possess grana but lack chloroplasts as well as nuclei. The chloroplasts of some desmids are very loosely organized, consisting of open networks capable of spontaneous changes in shape (23). Algal chloroplasts otherwise are exceedingly diverse with respect to size, shape, pigmentation and internal organization (24). In some algae and in all higher plants, the chloroplasts are lens-shaped. Chloroplasts of the latter type are readily isolated in the unbroken state, and have been used extensively in research. In comparison with chloroplast-bearing plants, blue-green algae and photosynthetic bacteria effect photosynthesis with similar quantum efficiencies (25, 26), photosynthetic capacities and growth rates (27 to 30), and their carbon assimilation reactions appear to be the same as in green plants (31 to 34). Chloroplasts are the largest intracellular structures associated specifically with photosynthesis, but since they are not always present, they cannot be essential. Their role in photosynthesis seems comparable to that of nuclei in heredity—organisms which exhibit photosynthesis without chloroplasts exhibit heredity without nuclei. Isolated chloroplasts have proved invaluable in research, but the mechanism of photosynthesis in chloroplast-bearing plants is not known to be superior in any way to photosynthesis in blue-green algae.

It is not always necessary or desirable to employ isolated chloroplasts in studies of water photolysis or of other parts of the photosynthetic mechanism. The use of whole algal cells in studies of the Hill reaction began with the work of Noack *et al.* (35) and of Warburg & Lüttgens (36), who also introduced the use of *p*-benzoquinone as an artificial oxidant. Quinone is well-suited for this application because of its penetrability and selective inhibition of dark synthetic reactions. Green (37 to 44), blue-green (43, 44, 45) brown and red algae (46) all have been used in Hill reaction studies of this kind. The isolation of chloroplasts introduces a number of problems which will be discussed consecutively. These are chloroplast isolation and purification, and problems arising from losses and derangements of water-soluble plastid constituents, natural inhibitors, and thermal deterioration. The situation is sometimes complicated by opposing requirements in these different categories which necessitate compromises, e.g., chloroplast purification by washing is apt to displace water-soluble constituents.

Chloroplast Isolation and Purification

The general method that is used in maintaining chloroplasts intact after their isolation was originated in the nineteenth century. Their purification involves fractional centrifugation, usually as described by Granick (47). Turgid leaf blades are macerated under several volumes of ice-cold 0.5 *M* glucose or other inert solutions of similar tonicity; unbroken cells are removed by filtration; whole chloroplasts are then separated from chloroplast fragments, heavier and lighter particles, and from the cell sap and soluble cytoplasm by fractional centrifugation near 0°C (47). Chloroplast fragments and

mitochondria sediment at *circa* 10 times higher R.C.F. than whole chloroplasts, but otherwise are recovered in the same way. Chloroplasts isolated in this way retain swollen nuclei (48), and chloroplast fragments are not separated from mitochondria, but these contaminants assume importance only in special studies. Some chloroplasts are always broken while the tougher cell walls are being ruptured, but Jagendorf & Wildman (48) have shown that less chloroplast breakage occurs during brief blendor treatments than when the leaves are ground in the usual way with sand. The severity of the required grinding treatment is determined by the toughness of the cell walls, and for that reason only easily ruptured cells such as those of tender leaves have proved satisfactory as sources of intact plastids.

According to McClendon (49), it has been known for a century that chloroplasts will swell and burst when placed in water, and it has been known for about seventy years that this can be prevented with concentrated sugar solutions. Meanwhile, these simple facts have been used in the majority of chloroplast studies that have been conducted, without exact knowledge of the forces that control chloroplast swelling. Assimilation of carbon dioxide within chloroplasts (5) necessitates an outgoing traffic of the photosynthetic products that are required elsewhere in the living plant. Respiration in mitochondria necessitates an inward traffic of substrates. Water-soluble products of photosynthesis and substrates of respiration (sucrose, glucose, etc.) nevertheless are the standard solutes used in preventing swelling in isolated chloroplasts and mitochondria. Simple salt solutions (NaCl, KCl) have also been used as chloroplast media, but no intrinsic advantages have yet been disclosed for salt versus sugar solutions.

Osmotic pressure gradients normally exist within individual leaves, whose chloroplasts at any instant are therefore subjected to a range of osmotic presures (50 to 53). It has long been known that the osmotic pressure of leaf sap varies diurnally, seasonally, with the position of the leaf on the plant, with the species and habitat, and with all other factors influencing intracellular supplies of water, solutes and colloids (50 to 53). For these reasons, the term "isotonic" rarely can be applied with precision to artificial media for leaf chloroplasts. In comparison with the chloroplast's fluctuating natural environment, the compositions and osmotic pressures of standard leaf grinding fluids (e. g.,0.5 *M* glucose) have been remarkably uniform.

The physical factors controlling chloroplast swelling have been investigated by Mercer *et al.* (54) with the aid of the electron microscope. Their studies indicate that swelling occurs mainly in the interlamellar region, that it is controlled by Donnan (gel) systems as well as by diffusible solutes, that the chloroplast is differentially permeable, not semipermeable, and that chloroplast swelling is irreversible (54). The physical interpretation offered by Mercer *et al.* accounts for the main characteristics of chloroplast swelling, but it may also be subject to biochemical influences. The swelling of chloroplasts and mitochondria may have the same basis (54); yet Fonnesu & Davies (55) found that osmosis does not account for swelling in liver mito-

XUM

chondria. Their swelling in very dilute sugar solutions was largely prevented by 10^{-3} *M* AMP, and was also reduced by 10^{-3} to 10^{-5} *M* DNP, by 4 m*M* KCN, and by anaerobiosis. Corresponding information on chloroplasts is lacking.

Jagendorf & Wildman (48) found that swollen nuclei remain with whole chloroplasts during their isolation by Granick's method. This contaminant was removed when the chloroplasts were passed through sharkskin filter paper, which acts as a selective adsorbent. Jagendorf (56) also has devised a flotation method for chloroplast purification. After their isolation by fractional sedimentation, the chloroplasts are suspended in glycerol-sucrose solution having a density that is just high enough to cause most of the chloroplasts to rise in the ultracentrifuge. With this method, young growing chloroplasts were found to have higher densities than mature ones, and chloroplasts in a single suspension commonly exhibited a range of densities (56). Thoroughly washed and purified chloroplasts contained no catalase or cytochrome oxidase, contained little nucleic acid, but effected the photochemical reduction of ferricyanide, *p*-benzoquinone, cytochrome C, and TPN (57). The reduction of DPN was not effected by purified chloroplasts (57).

The importance of water-soluble plastid constituents varies with the plant source and purpose of the research. Retaining such constituents in their natural "active" state can introduce requirements which conflict with those for chloroplast purification. The situation is simplest when chloroplasts of green plants are used in Hill reaction studies. Here the catalysts of water photolysis are all bound in the water-insoluble chlorophyll-lipoprotein, whose photochemical activity is insensitive to washing. The only water-soluble factors that must be supplied are chloride or related anions, and a buffered artificial oxidant. When the water-soluble phycocyanins and phycoerythrins (58) are present, their natural configuration in plastids and grana must be preserved *in vitro* for the retention of Hill reaction activity (45, 49). These colored protein micelles are displaced and eventually extracted when otherwise intact plastids and grana are isolated in the usual way with sugar or salt solutions (45, 49). The resulting bilichromoprotein solutions and chlorophyll-lipoprotein residues are then incapable of water photolysis, alone and in admixture. When present, phycocyanins and phycoerythrins form integral parts of the pigment-lipoprotein structures that effect water photolysis, and their loss causes physical gaps or other irreversible changes within the lamellae (45). The migration of these large protein molecules [mol. wt. *ca.* 300,000 (59)] during plastid isolation with sugar or salt solutions is instructive, since it provides visible evidence of a general problem. The dark reactions of CO_2 assimilation are now known to involve a large number of water-soluble catalysts and intermediates. The latter compounds are presumably dislocated and lost in the same way as the colloidal phycocyanins and phycoerythrins. When the water-soluble bilichromoproteins form part of the water photolysis apparatus, the Hill reaction seems to be as sensitive to plastid isolation as CO_2 assimilation by leaf chloroplasts.

Phycocyanins and phycoerythrins are retained in chloroplasts and grana when concentrated solutions of hygroscopic polymers [Carbowaxes (49) dextrins (45)] are employed as maceration media. Grana of blue-green algae (45) and chloroplasts of red algae (49) have exhibited high Hill reaction activity *in vitro* only when isolated with these viscous media. If Carbowax acted solely on the bilichromoproteins, it should not affect water photolysis in leaf chloroplasts. McClendon (49) observed higher and more stable Hill reaction rates in leaf chloroplasts, however, when these were isolated and tested in 30 per cent w/v Carbowax 4000 versus 0.5 *M* sucrose. This result was obtained consistently at the University of Minnesota, but not at the Hopkins Marine Station (49). Bishop *et al.* (60) observed no preservative action of polyglycols upon isolated leaf chloroplasts. Clendenning *et al.* (61) found that the stabilizing action of Carbowax upon leaf chloroplasts is exerted indirectly by way of cytoplasm, which is precipitated on the plastids by this reagent. When the cytoplasm is removed beforehand with 0.5 *M* sucrose, 30 per cent Carbowax 4000 no longer stablizes the Hill reaction (61). This finding seems to explain why Bishop *et al.* (60) observed no stabilization by polyglycols, cytoplasm having been removed in their studies prior to the stability tests. High reaction temperatures, lengthy storage at 0°C., and the use of limiting amounts of chloroplasts in the reaction vessels all provide the catalyst-limited condititions under which the stabilizing action of Carbowax-cytoplasm is strikingly evident (61). In a related investigation, Stocking (62) found that phosphorylase, catalase, and cytoplasmic protein is precipitated on leaf chloroplasts by the Carbowax solution that was employed by McClendon (49).

Carbowax also binds dissolved tannins, lessening their adsorption by chloroplasts and by collagen, and preventing their extraction from water with ethyl acetate (61, 63). The location and state of the tannins *in situ* determines the importance of this Carbowax property; the protective action of Carbowax against leaf tannins can only be observed on chloroplasts that have not been inactivated by tannins before being released into the grinding fluid.

The Hill reaction offers an instructive contrast to complete photosynthesis in its insensitivity to mechanical injury. In the absence of bilichromoproteins and natural inhibitors, high capacities for water photolysis are retained during the anaerobic fragmentation of chloroplasts to virus-sized particles at 0°C. (64). Sufficient information has accumulated to allow the effects of chloroplast isolation treatments upon complete photosynthesis to be followed more or less stepwise. Effects of mild cell deformation upon photosynthesis are apparently transitory: Willstatter & Stoll (65) showed that photosynthesis in leaves was scarcely affected by the removal of the epidermis or by cutting the leaves into 2 $cm.^2$ squares, treatments which would involve extensive "handling"; *Chlorella* that had been subjected to 145,000 R.C.F. for 3 hr. at 0°C. exhibited normal photosynthetic activity shortly after centrifuging (66). The latter treatment did not cause cell breakage (66), but would likely cause intracellular displacements of a reversible

XUM

nature (67, 68). Racusen & Aronoff (69) found that intact mesophyll cells isolated carefully from soybean leaves retained *circa* 20 per cent of the normal photosynthetic capacity. Tolbert & Zill (70) observed that whole protoplasts gently extruded through the cut ends of giant algal cells retained 12 to 15 per cent of the normal photosynthetic capacity for a short time. Merely stirring the extruded protoplasts reduced photosynthetic activity sharply (70). All of the foregoing treatments are very mild, of course, in comparison with those used in isolating chloroplasts. According to Willstatter & Stoll (65), the photosynthetic capacities of mature green leaves at 25°C. range from 2600 to 8500 mm.^{3} CO_2/hr./mg. chlorophyll. Initial capacities for the Hill reaction in chloroplast fragments freshly isolated from inhibitor-free species have ranged from *circa* 500 to 2500 mm.^{3} O_2/hr./mg. chlorophyll at 10°C. (71). Recent comparative studies of water photolysis, phosphorylation, and CO_2 assimilation in whole and broken chloroplasts have been yielding information of fundamental importance, but the capacity for CO_2 assimilation *in vitro* has been mainly in the range 0 to 20 mm.^{3} CO_2/hr./mg. chlorophyll (72). The progressive reduction in CO_2 assimilation capacity attending mechanical treatments of increasing severity is likely caused by derangements and losses of water-soluble catalysts and intermediates which play no part in water photolysis.

A freezing treatment used by Fager (73, 74) requires discussion, since it has been claimed to yield chloroplasts which were incapable of the Hill reaction, though capable of photosynthetic CO_2 assimilation. Arnon (5) and Lumry *et al.* (3) have indicated the difficulty of understanding this result. Fager (73, 74) froze leaves in liquid N_2, ground them to a fine powder, then suspended the powder in cold 0.05 *M* sorbitol borate, pH 6.9, containing 0.01 *M* KCl. Unbroken cells were removed by filtration. The resulting cell-free suspension contained the contents of the broken cells, diluted by the buffer. This crude chloroplast suspension exhibited "negligible" Hill reaction activity as a result of the preparatory freezing. When freezing was omitted, chloroplasts from the same batch of leaves exhibited high Hill reaction activity (73). This result was not surprising, since damaging effects of liquid air had been noted previously, and the preservative effect of concentrated sugar solutions had been apparent in less severe freezing treatments (71, 75). The next point concerns the degree to which the capacities for the Hill reaction and for CO_2 assimilation were retained in Fager's "frozen-and-thawed" chloroplasts. CO_2 assimilation *in vitro* amounted only to 0.1 to 0.2 per cent of photosynthesis in intact leaves (73). Fager did not state how he measured the Hill reaction, and comparable data were not provided concerning the retention of Hill reaction capacity versus CO_2 assimilation. The C^{14} method which was used in measuring CO_2 uptake can be made thousands of times more sensitive than manometry. Interpretation of Fager's results presents no difficulty if the methods used in measuring CO_2 uptake and O_2 evolution were of different sensitivities, as appears likely. Preparatory freezing was omitted in Fager's later work (76), and has not been used in other studies.

Natural Inhibitors

If the Hill reaction represents the water photolysis component of photosynthesis operating with an artificial oxidant, one might expect to observe it consistently in chloroplasts isolated in a standard way from any plant cells or tissues capable of photosynthesis. Chloroplasts possessing high Hill reaction capacities actually are obtained only from selected species, and the activity of chloroplasts freshly isolated from a single species often varies widely. Hill (16) and McClendon (49) attributed this phenomenon to physical factors [plastid rupture (16), unfavorable osmotic conditions (49)]. This type of interpretation does not account for several facts. Chloroplasts freshly isolated from the leaves of many species are consistently inactive when isolated intact in ice-cold sugar solution (77); chloroplasts having a high Hill reaction capacity when isolated intact retain this ability when finely disintegrated (19, 37, 64); the osmotic environment of chloroplasts *in vivo* is not constant, and their Hill reaction capacities *in vitro* are quite insensitive to osmotic pressure changes (78).

The first clear evidence of natural inhibitors of the Hill reaction was obtained by Kumm & French (77). They noted that inactive chloroplasts were often associated with tannins or related compounds which darkened ferric oxalate-ferricyanide solution. Clendenning & Gorham (71) demonstrated the presence of heat-stable water-soluble inhibitors in inactive chloroplast suspensions, whose action was irreversible and independent of the artificial oxidant. In a recent study (63), it was found that tannins and acids are the principal irreversible inhibitors of the Hill reaction which are brought into play during chloroplast isolation. Chloroplast inactivation by organic or mineral acids occurs from pH 4.2 downwards (63). The vacuolar sap of leaves commonly attains a pH below 4.0 (79), and there are several known "acid" species whose expressed sap has pH values below 2.0 (79). Chloroplasts isolated successively from leaves undergoing large diurnal changes in acidity can be expected to exhibit diurnal changes in Hill reaction activity. The acidity gradient within the pineapple leaf, as observed by Sideris & Young (80), in itself would result in the inactivation of chloroplasts isolated from the apex (pH 3.3) but not from the base (pH 5.5) of the same leaf. This type of chloroplast inactivation is caused by hydrogen ions; in the absence of other natural inhibitors, the inhibiting action of acidic leaf sap upon "active" chloroplasts is eliminated by prior neutralization (63). Use of strongly buffered grinding fluids provides little protection, since the chloroplasts are exposed to the acids during the intermingling of cell contents that precedes their release (63). Kinzel & Url (81) prevented the inactivation of enzymes during their isolation from acidic leaves by soaking the leaves beforehand in dilute ammonia; active chloroplasts presumably could be isolated from such leaves in the same way.

Tannins are common constituents of leaves (82) multicellular marine (83) and fresh-water algae (84). Little is known concerning their metabolism and function. There are many different kinds of tannins, which frequently occur as complex mixtures. Tannins are deposited in the leaf vacuoles, in

XUM

the cell walls, and in idioblasts. Tannin contents of leaves of a single species can vary sufficiently to cause chloroplast inactivation during isolation from one leaf sample and not another (63). When the vacuoles are rich in tannins, provision of tannin-binding agents in the grinding fluid does not prevent chloroplast inactivation; the chloroplasts are exposed to the tannins before being released (63).

Viruses which grow in chloroplasts have been shown by Spikes & Stout (85) to inhibit Hill reaction activity on a chlorophyll basis at both limiting and saturating light intensities. Their inhibiting action should be equally large *in vivo*. Detrimental effects of oxygen during chloroplast fragmentation in darkness (60, 64) represents another irreversible type of inactivation by a natural agent. Leaf constituents having reversible inhibiting effects upon the Hill reaction, e.g., triterpenoid saponins (63), are of common occurrence, but their effects vanish when the chloroplasts are thoroughly washed. When water photolysis remains linked *in vitro* with phosphorylation, CO_2 assimilation, nitrate reduction, etc., possible sites of action for natural inhibitors and destructive enzymes are multiplied.

Chloroplast sources have long been selected empirically, but the requirements can now be specified. These requirements seem to explain why the leaves that have become "standard" chloroplast sources (spinach, chard, etc.) have also been used extensivly in the human diet as "greens." A tender texture is advantageous in both applications, acidity promotes chlorophyll destruction during cooking, and the human taste-buds share the chloroplasts' sensitivity to tannins and acids.

Willstatter & Stoll (65) observed that when geranium leaves were pressed until the intercellular spaces became filled with sap, their capacity for photosynthesis was essentially eliminated [residual traces of activity were attributed to undamaged cells next to the veins which escaped the pressure treatment (65)]. This mechanical treatment, of course, was much milder than those used recently in extruding "active" protoplasts and in isolating "active" chloroplasts. Geranium leaf sap is highly acidic (pH 3.5 to 4.0) and also contains tannins (63). [Willstatter & Stoll (65) noted the acidity of the extruded sap.] Squeezing geranium leaf sap into the intercellular spaces would expose the chloroplasts to the acids and tannins even though they remained within the cell walls.

The conditions that are now employed in Hill reaction studies differ considerably from those used in Hill's pioneering studies (86, 87). The chloroplasts employed by Hill were incapable of oxygen evolution under the conditions now used: oxygen evolution ceased within 2 min. after intact plastids were ruptured (86), and ceased within 6 min. when the reaction mixtures were shaken (87). (For the past ten years, whole and fragmented chloroplasts have both been used, always with strong agitation.) The characteristics of Hill's chloroplast suspensions suggest the presence of natural inhibitors whose effects were aggravated by plastid rupture, but it would be necessary to investigate his chloroplast sources to settle this point with certainty.

Heterotrophic and autotrophic plants synthesize antibiotics, drugs and other "natural inhibitors" in great variety. There may be some overlapping between the inhibitors which act after cellular organization is destroyed, and those which act within or near undamaged plant cells, both upon their own metabolism and upon that of parasites and competitors. There are strong indications that the starch-sucrose equilibrium in plants is subject to control by natural inhibitors (88, 89, 90). The chemical nature of the latter inhibitors remains unknown. The acidity gradient within sugar beet leaves influences parasitism by leaf hoppers and by the virus they transmit (91). Tannins exert a powerful antibiotic action against viruses (92, 93). Conversely, photosynthesis in *Chlorella* is inhibited by the antibiotics Penicillin G (94) and Chlorellin (95). Photosynthesis *in vivo* is influenced by unidentified acidic inhibitors formed in the same cells (35), as Franck has pointed out (96).

Thermal Deterioration

When physically intact leaves are starved in darkness for long periods, they eventually lose their capacity for photosynthesis. Chloroplasts isolated successively from such starving leaves exhibit decreasing capacities for the Hill reaction on a chlorophyll basis (71). Living algae also exhibit gradually declining capacities for photosynthesis and the Hill reaction on a chlorophyll basis, as a result of storage deterioration *in vivo* (43). When photosynthetic cells are killed by grinding or with quinone, thermal deterioration of the water photolysis apparatus is thereby accelerated. Brown (43) has shown that Hill reaction activity is about equally thermolabile in isolated chloroplasts and in unbroken algal cells that have been killed with quinone. Thermal deterioration of chloroplast fragments has been investigated intensively by Bishop *et al.* (60). Their kinetic studies indicate that a second-order reaction is responsible for loss of activity at 0°C. At temperatures above 25°C., a first-order inactivation is also involved. The stabilizing action of "Carbowax-cytoplasm" is equally manifest during lengthy storage at 0°C., and during brief exposure to temperatures above 25°C. (61). Both of the inactivating reactions postulated by Bishop *et al.* (60) may, therefore, be inhibited by "Carbowax-cytoplasm" *in vitro* (61) as well as by living cytoplasm *in vivo*. Schwartz (97) has stabilized the Hill reaction capacity of whole *Chlorella* cells by snap-freezing over dry ice followed by lyophilization and storage at −15°C. Isolated chloroplasts are completely stabilized by frozen storage in concentrated sugar solutions near −40°C., but thermal deterioration occurs in frozen chloroplasts at −20°C. and higher temperatures (60, 75). The foregoing observations suggest that thermal deterioration involves hydrated chloroplast components. Inactivation at high temperatures shows high Q_{10} values and high activation energies (1), which are characteristics of heat denaturation of proteins. The chemical changes associated with chloroplast deterioration near 0°C. remain unknown. Loss of phospholipids is a possible causal factor, but it is not yet possible to relate changes in chloroplast phospholipids to changes in Hill

XUM

reaction capacity. Extensive data have been provided by Kates (98, 99) on the action of chloroplast phospholipase upon externally supplied substrates mobilized with ether. Characterization of chloroplast phospholipids is still incomplete, and no information is available on phospholipids and phospholipases in grana.

The Ontogenetic Factor

Development of photosynthetic capacity often lags behind chlorophyll synthesis, in growing leaves and during the greening of etiolated leaves (65, 100, 101, 102). Loss of photosynthetic capacity in senescent leaves has been observed to occur more rapidly than chlorophyll decomposition (65). The Hill reaction capacities of isolated leaf chloroplasts have been observed to undergo similar ontogenetic changes (71, 103, 104), which are not caused by natural inhibitors (71). The minimum apparatus for water photolysis, although small, is highly organized. Synthesis of the laminated chlorophyll-lipoprotein structures must involve many enzymatic processes, but how these are all integrated to produce organized lamellae remains unknown. Intermediate stages of their synthesis and destruction are apparently characterized by low capacities for water photolysis on a chlorophyll basis. The solution of this problem might greatly clarify the mechanism of water photolysis.

Further Characteristics of the Hill Reaction

The action spectrum for the Hill reaction in leaf chloroplasts was shown by Chen (105) to match the action spectrum for photosynthesis in green algae. Complete action spectra for the Hill reaction in red and blue-green algae are lacking, but it has been shown that phycocyanin (45) and phycoerythrin (46) will serve as light absorbers for the Hill reaction. In common with chlorophylls, these accessory pigments sensitize the Hill reaction only when located in the organized lipoprotein complexes. Energy transfer from accessory pigments to chlorophyll presumably occurs in the Hill reaction as in photosynthesis. Hill reaction activity is retained in very small chloroplast fragments (19, 37, 64), but internal organization must be preserved in these particles (10, 45, 106). The minimum quantum requirement for the Hill reaction at low light intensity appears to be the same as for photosynthesis (39, 107), but the light intensity required to saturate the Hill reaction is usually higher (38, 43). The thermolability of Hill reaction systems narrows the temperature range that is suitable for their study, but at low temperatures, their activation energy is 8 to 10 kcal. (60). The maximum rate of oxygen production was observed to be the same for photosynthesis and the Hill reaction in *Chlorella* at 10°C. (38, 39). *Chlorella* (108) and chloroplast (56) suspensions are both heterogeneous; no information is available on the Hill reaction characteristics of the separable fractions in *Chlorella* and chloroplast suspensions.

Chloroplasts from inhibitor-free sources serve as electron-donors when

illuminated in the presence of suitable electron acceptors. The great variety of acceptors that have been used shows that this requirement is nonspecific. (The artificial oxidant presumably maintains the catalytic oxidant of chloroplasts in the oxidized or "acceptor" state.) The Hill reaction capacity of leaf chloroplast fragments is insensitive to washing, and also remains high on a chlorophyll basis when the chloroplast concentration is greatly reduced in the reaction vessel. Uri (109) and Wessels (110) obtained no evidence of free radical intermediates in the Hill reaction. These observations indicate that the catalysts and intermediates of water photolysis are all bound in the water-insoluble chlorophyll-lipoprotein; presumably the labile intermediates indicated in recent kinetic studies (3, 40, 111) are bound to the catalysts. The organization of chlorophyll in films within the layered lipoprotein seems ideally suited for the transfer of absorbed light energy to localized catalytic centres, for which there is extensive indirect evidence. If each chlorophyll molecule could effect water photolysis, the maximum capacity for oxygen production should be about one half of the chlorophyll molarity per catalytic working period. Actually it is 1000 times less, in the Hill reaction as well as in photosynthesis (39). Certain derivatives of dimethyl urea inhibit the Hill reaction reversibly when present in only one hundredth of the chlorophyll concentration (112). A number of bound redox systems are known to be present in washed chloroplasts in very much lower concentrations than chlorophyll [cytochrome-*f* (16, 17, 117), manganese (114), 6,8-thioctic acid (115), and vitamin K (116)]. Corresponding information on grana is lacking. The foregoing observations support the view that the catalytic centres of water photolysis are far less abundant than chlorophyll molecules, in conformity with the Emerson-Arnold unit concept. A minimum of two catalytic sites (or two sets of catalysts) seems to be required at each centre, one serving in the transfer of hydrogen from water to the soluble oxidant, the other serving in oxygen formation. Means must also be provided for the prevention of back-reactions between the oxidizing and reducing intermediates of water photolysis, except in the coupled phosphorylation process of photosynthesis (5).

Redox Catalysts of the Chloroplast

Cytochrome-*f* is present in *circa* 1/400th of the chlorophyll concentration in chloroplasts (16, 17, 113), and is associated specifically with photosynthesis in plants (16, 17). Its oxidized spectrum appears immediately after isolated chloroplasts, leaves, green, and red algae are illuminated under oxidant-deficient conditions (113, 117). Its spectral response to illumination and high oxidation potential suggest its association with an oxygen-rich intermediate [cytochrome-*f*-OH? (40)]. Catalysis of the oxygen-liberating step by bound manganese is now clearly indicated: photoreduction in hydrogen-adapted algae was found by Kessler (118) to be insensitive to manganese deficiency, whereas photosynthesis and the Hill reaction were found highly sensitive to manganese by Eyster *et al.* (119). Manganese bound in the chloro-

XUM

plast protein fraction was not removed by exhaustive extraction with water or with fat solvents (114). The postulated role of 6,8-thioctic acid in water photolysis is now in hydrogen transport (9). The stimulating effect of assimilated thioctic acid upon the Hill reaction in quinone-limited *Scenedesmus* (41, 42) can be satisfactorily interpreted on this basis. The Hill reaction is insensitive to sulfhydryl poisons (110, 120), which has been used as evidence against the participation of thioctic acid (120). The sensitivity of sulfhydryl systems to these poisons is highly variable (121), so thioctic acid cannot be excluded as a catalyst of water photolysis on the basis of the inhibitor evidence. Dam (116) and Wessels (112, 122) have marshalled extensive evidence in support of the view that bound vitamin K is a catalyst of water photolysis. The bound redox systems of chloroplasts are all in need of further study, especially in "minimum particles" and in chloroplasts undergoing ontogenetic changes in Hill reaction capacity. Information appears to be entirely lacking concerning cytochrome-*f*, manganese, thioctic acid, vitamin K and other catalysts in the grana of blue-green algae, and concerning their relative abundance in the grana and stroma of chloroplasts.

Quantum Conversion

The quantum conversion mechanism of photosynthesis and the Hill reaction presents conceptual difficulties because so many quanta are required to produce each oxygen molecule. Oxygen formation is preceded by the photolysis of two water molecules, and the extraction of each oxygen "atom" involves the rupture of two oxy-hydrogen bonds, which cannot be effected with a single red quantum. All of the required energy must be provided via absorbed light, directly or indirectly. The minimum quantum requirement imposed by the heat of formation of water is one quantum per oxy-hydrogen bond ruptured, two quanta per water molecule consumed, or four quanta per oxygen molecule evolved. The minimum quantum requirement indicated by experimental evidence for photosynthesis and the Hill reaction seems to be double these values. Observed quantum requirements for the Hill reaction include extraneous losses as heat (39, 107), and those for complete photosynthesis also include the light that is harnessed via phosphate bond energy (5, 8, 9, 11).

The way in which several light quanta cooperate in the photolysis of individual water molecules, and the way in which light-activated chlorophyll cooperates with the catalytic centres of water photolysis remains unknown. One type of hypothesis envisages the photolysis of water as a one-quantum triggered reaction, extra energy being supplied as recycled chemical energy (recycling of chemical energy as water-soluble compounds is excluded, however, in the Hill reaction). Hypothetical mechanisms of this category seem to have been eliminated by research that is discussed elsewhere (2, 3, 9, 11). A second type of mechanism has been suggested by Allen & Franck (40); the energy of two successively absorbed quanta is stored as physical energy in a single chlorophyll molecule; the second quantum raises chlorophyll from the

metastable state to an "excited triplet state" which would confer sufficient energy for water photolysis in a single photochemical reaction. The time interval over which the first quantum would have to remain in the metastable state at very low light intensities, however, greatly exceeds the expected lifetime of this physical state. It has long been assumed that the oxygen of water is raised to a "photoperoxide" state in water photolysis so that oxygen can be eliminated by a dark reaction [Photoperoxide dismutation by Franck & Herzfeld's catalyst "C" (123).] Existing evidence allows other possibilities. The author has suggested (10) that the two oxy-hydrogen bonds of water are broken photochemically by two successive one-quantum reactions (photochemical cleavage of bound water followed by photolysis of the bound hydroxyl derivative). Flashing light studies of the Hill reaction by Gilmour *et al.* (111) and by Allen & Franck (40) indicate that intermediates of water photolysis survive longer than known forms of electronic excitation energy. By viewing these bound intermediates as substrates of further one-quantum reactions, a feasible working hypothesis is obtained for the multiple quantum mechanism of water photolysis.

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AUXIN RELATIONS IN ROOTS[1,2]

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Introduction

In the early thirties a beautifully integrated picture of the auxin regulations of root and coleoptile growth was seemingly obtained. The tips of both types of organs were thought to produce or to activate the auxin, which was polarly transported to the zone of cell elongation. Here it caused a stretching of the cell walls of the coleoptile proportional to the amount present; in intact roots the amount of auxin was thought to vary within the supra-optimal range, an increase causing a retardation of the cell elongation. The different results of a moderate decapitation (a rapid decrease of the growth rate of the coleoptile and a slight stimulation of root elongation) were easily understood on this basis, as were the different geotropic responses occurring as the result of a downward transport of auxin in horizontally placed organs under the influence of gravity. A basic necessity of auxin even for the elongation of root cells was fully compatible with this picture, a condition for its demonstration being the attainment of roots or root tissue with an auxin content well below the optimal value. The obvious way to accomplish this was the use of decapitated roots or root sections.

The situation would be fundamentally changed, however, if even intact seedling roots with normal geotropic reactions were found to show accelerated longitudinal growth when treated with highly diluted auxin solutions. Such behaviour has been reported especially for cress roots, and the stimulations have even been used as a criterion by which true auxins could be differentiated from inhibitors with retarding effects only upon root growth [Pohl (122); Ochs (115)]. In some cases the reported auxin stimulations of intact roots cannot be reproduced under identical or slightly different experimental conditions, and in other cases the stimulations seem to appear only after the disappearance of the applied auxin from the medium. Such after-effects may be interpreted as an "adaptation" of the roots to higher auxin levels, and so are compatible with the classical view of the auxin regulation of root growth. That low concentrations of a typical auxin (e.g., IAA) should be able to stimulate the growth of intact seedling roots, when still maintained in the solution surrounding the root, has not been conclusively proven.

Hence, the classical concept of the auxin regulation of root growth still seems to be valid, and the present review will, to some extent, be an attempt

[1] The survey of the literature pertaining to this review was concluded in August, 1956.

[2] The following abbreviations will be used: IAA, 3-indoleacetic acid; NAA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; 1-NMSA, 1-naphthalenemethylsulfide-acetic acid; 1-NMSP, the corresponding α-propionic acid.

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to consider critically the apparent contradictions and, if possible, to resolve them.

The Occurrence of Auxin in Roots

Good evidence for the presence of growth regulators of similar or identical type in roots and in coleoptiles was presented by Cholodny who showed that decapitated corn roots (*Zea mays*) were able to perform normal geotropic reactions when a corn coleoptile tip was placed on the cut surface (50). The longitudinal growth of such roots was retarded to 64 per cent of the growth of decapitated control roots. On the other hand corn root tips placed on decapitated oat coleoptiles (*Avena sativa*) accelerated their growth and restored their ability to perform almost normal geotropic and phototropic curvatures (51). Also intact corn roots were strongly inhibited by apically placed corn coleoptile tips, but not by such tips placed 4 to 5 mm. from the root apex (53). Acceleration of the growth of decapitated oat coleoptiles or inhibition of decapitated lupine roots (*Lupinus angustifolius*) was obtained only by the use of root tips (corn, lupine), not by sections from the stretching zone of the roots (52).

The more sensitive method with root tips placed asymmetrically on the cut surface of decapitated oat coleoptiles was first used by Boysen-Jensen (37) who obtained positive results with tips from roots of corn and broad bean (*Vicia faba*). The auxin can also be collected in agar which must, however, contain 10 per cent glucose to give the best results. Auxin from broad bean and corn root tips had earlier been collected in pure gelatine (10 per cent), and had been found to cause positive curvatures when placed eccentrically on the cut surfaces of decapitated roots [Hawker (74); Snow (137)]. The amount of auxin obtained from root tips with Boysen-Jensen's method was even higher than that obtained from oat coleoptile tips (37). Also from subapical root sections auxin could be obtained, though in amounts rapidly decreasing with the distance from the tip. After 6 mm. they were practically nil. Similar results have been reported for corn and broad bean roots by Gorter (69) and for *Cucurbita* roots by Nagao (109).

A somewhat different picture of the auxin distribution in roots is obtained with the chloroform extraction method of Thimann (149). The decrease with growing distance from the tip is still apparent though much weaker, considerable amounts being present in oat roots (149) more than 20 mm., and in broad bean roots [Fiedler (61)] more than 30 mm. from the tip. For the latter type of roots Kefford (84) found sections taken 0 to 20 mm. and 20 to 40 mm. from the tip to yield amounts of IAA and other growth regulators which did not differ significantly (extraction with absolute alcohol, chromatography).

The amounts of auxin extracted from oat roots per mm. were of the same order of magnitude as those extracted from the coleoptile (149). Also the discrepancy between the appreciable auxin amounts obtainable by chloroform or alcohol extraction of the basal parts and the absence of auxin free

to diffuse out into agar from these parts is the same for roots and coleoptiles. The explanation of this phenomenon is not clear, but it might possibly depend on a much more efficient delivery to the agar of auxin under active transport from the centres of production by means of the polar mechanism than of auxin already disengaged from this mechanism [cp. Went & Thimann (159)]. A slow diffusion of such auxin out of the older tissues might easily be masked by inactivation processes at the cut curfaces. To this may be added the possibility that considerable amounts of the easily extractable auxin may be located, and tenaciously retained, in the vacuoles of living cells (see below).

The determination of extracted auxin is complicated by the presence of substances interfering with the auxin effects in the biological tests. Crude ether extracts of *Armoracia* roots were thus found by Boysen-Jensen (42) to lack auxin effects. After purification by diffusion an appreciable amount of auxin was found, though the purity was still not high enough for a quantitative estimation. Under the circumstances the introduction of chromatographic methods for separation of the growth regulators present in the extracts was a needed improvement, and a firmer ground for the study of the auxin contents of roots has already been laid by the work of Bennet-Clark and Kefford (30, 84), of Lexander (93), and of Audus & Thresh (24).

The acid fraction of an alcohol or ether extract made from roots of broad bean, pea (*Pisum sativum*), corn, and wheat (*Triticum vulgare*) was, by means of biological tests, shown to contain at least three types of growth-regulating substances. The highest activity usually occurred in the same place as IAA on the chromatographs, and there is little doubt as to the identity of the native substance with IAA. It invariably promotes the growth of coleoptile sections, and has been shown to occur in broad bean roots in a concentration inhibitory to the extension of pea root sections (84). Also in wheat roots it certainly occurs in amounts supraoptimal for the cell elongation when applied from outside. IAA from 8 to 27 gm. of fresh wheat roots occurred on two contiguous 2 cm. zones of the chromatogram, each of them being placed in 4 ml. nutrient solution, and each causing an inhibition of about 70 per cent in the cell length of wheat seedling roots (93). The IAA concentration of the eluate may thus have been up to three times higher than that of the fresh tissue, but in order to pass from such a pronounced inhibition, probably corresponding to at least 10^{-7} M, to the range of very small or no inhibition the eluate must certainly be diluted at least 100 times.

An "accelerator α," moving slower than IAA on the chromatograms and stimulating both coleoptile section, pea root section, and wheat root growth at concentrations roughly corresponding to that of the extracted tissue, was also found. It has been tentatively suggested that this substance might be 3-indolepyruvic acid [Stowe & Thimann (139)], but Housley & Bentley (75) interpret it as a neutral substance of unknown chemical nature.

A third growth-regulating substance found in roots moves more rapidly than IAA, and is inhibiting both to coleoptile section growth and to root

XUM

elongation. The chemical nature of this "inhibitor β" is as yet wholly unknown.

It is an important feature that the chromatographic studies have shown a "great similarity between the growth-substance contents of seedling roots and etiolated shoots" (84). As pointed out by Kefford (84) this similarity "is unexpected unless the physiological reactions of shoots and roots with the growth substances are opposites."

A rather different view of the growth-regulating substances of roots has been presented by Britton, Housley & Bentley (44). Working with intact and isolated tomato roots (*Lycopersicum esculentum*) and extracting them by a method which "would cause the destruction of any IAA present," they obtained rather irregular results, which nevertheless led them to suspect "that a non-indole system may be operating" in these roots.

Only few data on the absolute amounts of rapidly extractable and chromatographically purified auxin in roots are yet available, and a discussion of an indirect way for the estimation of the physiologically active concentration of auxin present in them may therefore be appropriate. Upon the assumption that the concentration of "free auxin" in the root determines the degree of saturation of the sites active in growth regulation, and that it easily equilibrates with auxin applied in the external solution, tentative conclusions might be drawn from the concentration-response curves for root growth inhibition. The first perceptible inhibitions would be caused by auxin concentrations just above the native ones. In this way Seiler (134) estimates the native auxin concentration of corn seedling roots as equivalent to about 10^{-9} M IAA, and Lundegårdh (100) concludes that the same value approximately corresponds to the concentration of native auxin in the surface layer of wheat roots. For pea roots a similar value would be deduced [Åberg & Jönsson (8)], while for flax seedling roots (*Linum usitatissimum*) a value about 10 times lower than that of wheat roots would be assumed [Åberg (6)].

There are naturally several sources of error in such estimations. An accumulation of auxin in the "free" state from the surrounding solution might be possible, especially at the very low concentrations causing incipient inhibition of root growth. The delimitation of "free auxin" from other forms may be difficult to perform; the existence of auxin gradients in the roots complicates the situation, and so on. An argument against the validity of the method may be obtained from the results of Lexander (93), referred to above. In this case, the amount of easily extractable IAA, when present in a solution volume corresponding to that of the fresh tissue, would probably inhibit the elongation of wheat root cells conspicuously.

Incredibly high auxin concentrations, about 10^{-3} M, were reported for cress roots (*Lepidium sativum*) by Moewus *et al.* (107). The methods used for their determination have, however, been justly criticized [see Reinert (127)]. Concentrations up to the same value have been reported for lentil roots (*Lens culinaris*) by Pilet (117, 119). The auxin was obtained by chloro-

form extraction during one hour and determined without purification by a modification of the *Avena* curvature test which seems to be of a quite extraordinary type. For *Brassica oleracea* roots Linser & Maschek (97) report an auxin concentration equivalent to 2×10^{-4} *M* IAA when estimated biologically, while the colorimetric determination of IAA gave 5×10^{-4} *M*. In the shoots the former value was much higher than the latter, presumably indicating the presence of auxins not related to indole.

Strongly contrasting to the values now cited are the data obtained by Audus & Thresh (23, 24) for pea roots. After extraction with ethanol at 0°C. and chromatographing they found about 1 μg. of IAA per kg. fresh weight of 7 to 12 days old pea roots, a figure approximately corresponding to an average concentration in the tissue of 6×10^{-9} *M*.

If the very high amounts of easily extractable auxin in roots reported by the first-mentioned authors should turn out to be correct, it becomes urgent to find some way to reconcile them with the high sensitivity of roots to externally applied concentrations 1000 or 10,000 times lower. No adequate data on the uptake of auxin in fibrous roots are available, but experiments on the IAA uptake in different other tissues have invariably failed to show an accumulation of extractable IAA in concentrations higher than those of the surrounding solutions [*Nitella* cells: Albaum *et al.* (10); *Cucumis* hypocotyls: Sutter (144); pea epicotyls: Andreae & Ysselstein (15); carrot disks (*Daucus carota*): Reinhold (128)]. Internal concentrations in excess of the external one may apparently be built up, but IAA is then accumulated in some bound form [protein complexes, indoleacetylaspartic acid, indoleacetamide (cp. 15)]. Another picture has, however, been obtained from experiments on the uptake of labeled 2,4-D into oat coleoptile sections [Johnson & Bonner (77)]. From 4.5×10^{-7} *M* solution 2,4-D was accumulated during 6 hr. to a concentration of about sevenfold, while the accumulation from a 9×10^{-6} *M* solution was only threefold. The accumulated 2,4-D was extracted and chromatographed, and all of the radioactivity present in the tissue was recovered in a spot corresponding to known 2,4-D. The absence of accumulation of free IAA in the studies mentioned may be related to the higher external concentration used (5.7×10^{-5} *M* or more), but possibly also to the more rapid metabolism of IAA.

The uptake of 2,4-D appears to involve at least three different processes (77): (*a*) diffusion into the tissue, (*b*) exchangeable binding in the tissue, and (*c*) continued uptake. The relation of these three processes to the auxin-induced growth processes is not clear, but Johnson & Bonner (77) suggest that in a sense "it appears to be the external auxin concentration rather than the total internal concentration which determines the growth rate of the coleoptile section. It is possible that the bulk of the auxin taken up by the tissue merely accumulates in the vacuole, as is the case with inorganic ions." The same may be valid for roots, and the possible presence of relatively large amounts of "free" but physiologically inactive auxin in the vacuoles would then be the solution of the apparent discrepancy between the

XUM

extreme sensitivity of roots to applied auxin and the high amounts of native auxin reported to be present in them. The amount of easily extractable "free" auxin would lack physiological significance in itself and would have to be divided into two parts, one with free access to the sites active in growth regulation, and the other isolated from them in the vacuoles.

The presence in oat roots of substances from which auxin may be liberated through enzymatic processes has been demonstrated by Thimann & Skoog (155) by means of prolonged ether extraction. In the pineapple plant (*Ananas sativus*) the production of auxin from precursors was studied for different types of tissues and found to be highest in young roots, which also contained the highest amounts of a neutral auxin precursor, presumably 3-indoleacetaldehyde [Gordon & Nieva (67)]. Relatively high concentrations of this substance were also found in other types of young roots (*Taraxacum officinale, Cichorium, Musa sapientium*). The presence of considerably higher amounts of such a neutral auxin precursor than of acid auxin has been confirmed for *Cichorium* roots by Warmke & Warmke (158) and for *Brassica* roots by Bentley & Bickle (32).

The Production of Auxin in Root Tips

A sustained flow of auxin from glucose-fed root tips has been shown both directly by means of negatively curving oat coleoptiles [Cholodny (54)] and indirectly by collecting the auxin in agar [Thimann (149); Nagao (109); Raalte (124)], and such a flow seems in itself to be a good indication of a continued production (or activation) of auxin in the tips. The rapid decline of the auxin content in decapitated broad bean roots [Boysen-Jensen (40)] lends support to this view.

A more strict proof of the production is, however, given by the demonstration that the auxin amounts obtainable by extraction are much lower than those collected in agar under favourable conditions during a period of some hours (109, 40), and by the fact that the auxin amount in root tips plus agar increases considerably over that originally present in the root tips (124). The rate of auxin delivery from the root tips to glucose agar increases during the first 2 to 3 hr. [Gorter (69), Raalte (124)], a phenomenon which likewise seems hard to explain upon other assumptions than a production of auxin in the tips.

The biochemistry of auxin formation has been studied mainly for other plant parts than roots [see Gordon (66)]. The effects of the possible auxin precursors and intermediate products upon root growth are, however, of some interest in this connection.

Tryptophan causes an inhibition of cress root growth to 50 per cent at a concentration of about 5×10^{-4} M [Audus & Quastel (20); Clauss (55); Jerchel & Staab-Müller (76)]. Its activity is at least 1000 times less than that of IAA, and nothing is known about the nature of the inhibition. As tryptophan may possibly exert a non specific toxicity at the relatively high concentrations needed for root growth inhibition, conclusions as to a con-

version to IAA cannot yet be drawn. The same holds true for tryptamin inhibitions (20). A reported stimulation of isolated rye roots (*Secale cereale*) by tryptophan (and IAA) turned out to be caused by an unidentified product formed from it during autoclaving [Roberts & Street (129)]; aseptically added, nonheated tryptophan was of very low activity.

3-Indoleacetaldehyde prepared by the tryptophan-isatin reaction was about 10 times less active than IAA in inhibiting the elongation of seedling roots of *Artemisia absinthium* [Ashby (16)]. Juice obtained from the roots had the capacity to convert 1-naphthaleneacetaldehyde to 1-naphthaleneacetic acid, and it seems rather probable that the inhibiting effect of 3-indoleacetaldehyde is attributable to the analogous conversion to IAA. The high activity of indoleacetaldehyde in root growth inhibition has been confirmed by Bentley & Housley (33). Using a sample of the pure compound they found it to be 5 to 10 times less active than IAA in the inhibition of cress seedling roots. The absence of significant stimulating effects of indoleacetaldehyde upon cress roots [Pohl (122)] can hardly be taken as an argument against such a conversion, as the stimulations caused by IAA are themselves rather dubious. Ashby (16) reports a slight stimulation of *Artemisia* roots at a concentration of indoleacetaldehyde 3×10^5 times lower than that giving 50 per cent inhibition. This stimulation may certainly have been related to sources of variation other than those caused by the different rate of growth of individual roots, and its physiological significance is doubtful (see further below).

3-Indoleacetonitrile is known to be converted to IAA in wheat and oat tissues but not in pea tissue [Thimann (152); Fawcett *et al.* (60)]. Analogously, it inhibits the root growth of germinating oats almost as effectively as IAA [Bentley & Bickle (32)], while the inhibiting effect on cress roots is 60 to 100 times less than that of IAA (32, 76). For excised tomato roots the concentration of the nitrile needed for 50 per cent inhibition of the linear growth of the main axis is even 1000 times higher than in the case of IAA. As the type of growth is in some respects different for roots inhibited by indoleacetonitrile and for those inhibited by IAA to the same degree, the nitrile may be assumed to have some effects of its own, irrespective of its possible conversion to IAA [Street *et al.* (142)].

No direct experience of the effects of 3-indolepyruvic acid on root growth seems to be at hand, which is deplorable with respect to its possible identity with "accelerator α." This latter substance increases the growth of oat coleoptile cylinders, the growth of pea root sections [Kefford (84)], and the final length of wheat root cells [Lexander (93)]. Indolepyruvic acid is known to give positive auxin effects in the *Avena* curvature test and in the pea test [Kögl & Kostermans (86); Haagen-Smit & Went (72)] which may be caused by a conversion to IAA. Antiauxin effects of the indolepyruvic acid molecules in wheat roots do not seem improbable, but may be difficult to study because of the tendency of the pyruvic acid to spontaneous breakdown [Gordon (66)].

XUM

Summarizing it may be said that an IAA synthesis from tryptophan is not yet proven for root tissues. Indoleacetaldehyde is probably converted to IAA at a rate sufficient to permit the appearance of auxin effects on root growth. The presence of relatively high amounts of a neutral auxin precursor, possibly the aldehyde, in young pineapple roots, and the close relation between the amount of this precursor and the auxin production during ether extraction [Gordon & Nieva (67)] is of great interest in this connection. There is good reason to assume that 3-indoleacetonitrile may likewise be converted to IAA in some types of roots, but not in others. A role of the nitrile in the normal auxin synthesis of roots is therefore not very probable. The possible role of 3-indolepyruvic acid in roots must be the object of further studies.

The Effects of External Factors on the Auxin Production and Content of Roots

Glucose.—The effect of glucose on auxin delivery from root tips to agar was first held to be caused by an osmotic suction, but the possibility of applying the glucose apically to the tips [Cholodny (54)] or of keeping the tips in a glucose solution before putting them on agar [Raalte (124)] shows that the effect must be mainly conditioned in some other way. In broad bean root tips suspended for 2 to 3 hr. in 10 per cent glucose solutions the oxygen consumption was decreased to about 60 per cent of the control rate, the carbon dioxide production was not significantly altered, and the extractable auxin content was about trebled. A change from air to pure oxygen increased the respiration strongly and lowered the auxin content of the tips. In the presence of a cyanide concentration inhibiting the oxygen consumption to about 60 per cent the auxin content of the root tips was never decreased, but sometimes increased. It was suggested (124) that the most probable explanation of these results is a decreased oxidation of auxin under conditions which result in a lower respiration rate. The increased delivery from glucose-fed root tips might then partly be connected with a decreased destruction of auxin, revealing a production which is also going on in the unfed tips. The destruction of auxin on the cut surface is, however, not influenced, as appears from the absence of conspicuous effects during the first hour (124).

Phosphate.—The amount of extractable auxin in broad bean root tips is also increased by phosphate buffers (124). In this case the oxygen consumption is unaltered, and the mechanism underlying the increase is completely obscure.

Nitrogen.—The production of auxin in shoot tips of *Helianthus* and *Nicotiana* [Avery *et al.* (25)], in the cotyledons of *Raphanus sativus* and in the coleoptile tips of *Zea mays* [Gorter (70)] as studied by the diffusion technique seems to be strongly increased with increasing nitrogen supply. Venturing the hypothesis of a corresponding relation between nitrogen supply and auxin production in roots, Söding (138) suggested the increased auxin production as a possible cause of the increased shoot-root ratio under the in-

fluence of high nitrogen supply. No direct evidence as to the effect of nitrate on the auxin production in root tips is yet at hand. Low-nitrogen wheat roots are, however, more sensitive to auxin applications (inhibition of growth) than are high-nitrogen roots, and they show much slighter growth stimulations from application of the antiauxin *p*-chlorophenoxyisobutyric acid than do the latter ones, phenomena which might both be expected as the result of a lower auxin level in the nitrogen-deficient roots [Bosemark (36)].

Light.—Very little is known with certainty on the effects of light on the auxin content of roots. It seems probable that ordinary daylight may increase the auxin production and the auxin content of detached *Zea* and *Helianthus* roots [Segelitz (133); Naundorf (112)]. On the other hand Pilet (117, 118) claims to have shown a lowering of the auxin content of lentil roots upon illumination with ultraviolet radiation ("lampe de Wood," $\lambda = 360 \pm 5$ mμ). See, however, further under the heading Phototropism.

Growth regulators.—A supposed indirect action of 2,4–D resulting in altering the level of native auxin in the plant has newly been disproved by Audus & Thresh (23, 24). No significant effects of 2,4-D-treatment on the content of endogenous growth regulators of pea roots were found, and the authors find little reason to doubt that 2,4-D is an auxin in its own right acting directly on the growth system.

The much-discussed action of 2,3,5-triiodobenzoic acid has received clarification by the demonstration of the capacity of this substance to lower the IAA content of pea roots drastically during a six to nine day treatment with relatively high concentrations (2×10^{-5} *M* and higher) (23, 24). Apparently this effect is most easily explained in connection with the antagonistic effects (or with part of them) of the triiodobenzoic acid, while the auxin synergism exerted by low concentrations both in coleoptile and in root growth [see 3, 8] remains enigmatical.

Pilet (119) found no effect of a maleic hydrazide treatment of lentil roots, while Audus & Thresh (23, 24) obtained distinct indications of a raised IAA level in pea roots. They concluded, however, that this raised IAA level may be purely incidental to the inhibition of the growth and may have little direct part to play in its cause (24).

The Transport of Auxin in Roots

Already the possibility of collecting auxin from excised root tips by placing them with the cut surface on agar shows that there must be an efficient basipetal transport in the young root tissues. Auxin is obtained only from the basal surface of tip sections from broad bean roots [Gorter (69)], and experiments with auxin externally applied to one of the cut surfaces of young root sections have repeatedly indicated a pronounced basipetal polarity of transport [broad bean: Nagao (109), Amlong (13); coleus: Leopold & Guernsey (90)]. Cholodny (54) found that excised corn root sections were inhibited in their longitudinal growth and regained their ability

to react geotropically under the influence of corn root tips only when these were placed on the apical end, and not when they were placed on the basal end of the root section.

With respect to the possibility that the polarity may be lost in root sections, especially under the influence of high auxin concentrations applied externally, experiments with intact roots seem of high value. Cholodny (53) placed three coleoptile tips apically on intact corn seedling roots, which responded with growth retardation and the production of swellings. When placed 4 to 5 mm. from the root apex, however, the coleoptile tips did not influence the growth of the roots appreciably. Jost & Reiss (80) found that IAA applied to the tips of corn roots was about 10,000 times more effective in inhibiting their growth than IAA applied 10 to 15 mm. from the apex.

In view of such positive evidence for a pronounced polarity of auxin transport in the apical parts of broad bean and corn roots, results indicating a complete absence of polarity in the same type of material [corn: Gorter (68); broad bean: Faber (59); cp. however Amlong (13)] must be considered dubious.

The polarity of auxin transport seems to be less pronounced in the more basal root parts. That it may sometimes be restricted to the very apex (1 to 2 mm.) appears from Syre's (146) results with corn and broad bean roots. On the other hand, Leopold & Guernsey (90) found predominance of the basipetal transport to be apparent also in the basal parts of coleus roots. In older root parts an auxin stream coming from the shoot and advancing towards the root apex through the cambium is rather probable [Snow (137); Söding (138)], and a phloem transport directed toward the apex must also be taken into consideration. Perhaps the inhibition of the growth of oat seedling roots under the influence of auxin application to the endosperm or scutellum may be related to a transport of the latter type. IAA applied to the scutellum gives such an effect according to Thimann (150), and Bonner & Koepfli (34) report an inhibition by application of IAA to the endosperm which was even stronger than by application to the root tip only.

Binding and Destruction of Auxin

Binding.—The idea that auxin may become bound to plasmatic sites inactive in growth regulation, and that such binding may be quantitatively preponderant, especially at unphysiologicallv high auxin concentrations, has been emphasized by Gordon in his recent review (66); cp. also Galston (62). This binding may be of a relatively loose type, or it may result in a protein complex of high stability. A complex of the latter type forms in pea roots kept in IAA-solutions (10^{-6} *M* or higher concentrations), presumably by an adenosinetriphosphate-dependent mechanism [Siegel & Galston (135)]. The newly found formation of indoleacetylaspartic acid in the same material [Andreae & Good (14)] makes it natural to assume that IAA is bound by a peptide linkage in the protein complex also.

No evidence for the formation of a corresponding Salkowski reactive protein complex has been found for etiolated pea epicotyls [Andreae &

Ysselstein (15)] and the possibility exists that it may be more or less specific for root tissues. In this connection the histochemical data of Ebert (57) are of interest. Seedlings of different types, pea among others, were placed in 2.86×10^{-3} *M* IAA during 10 hr., and were then found to show strong colorations with IAA reagents mainly in the root tissues (and for example in the growing zone of *Cucumis* hypocotyls). The IAA content was not appreciably influenced by daylong treatment with flowing water, and it may well have been present in the form of stable protein complexes. Conclusions as to the binding of auxin to the sites active in growth regulation seem scarcely warranted.

Recently Libbert (94) suggested that IAA may react enzymatically with a substance present in roots and green leaves, thereby producing a neutral, ether soluble but benzene insoluble inhibitor, which is present both in roots and shoots, and which may easily be hydrolyzed with liberation of IAA. The appreciation of the physiological role of this inhibitor in root growth (95) would be greatly facilitated by knowledge of the concentration-response curve over a wide range and of its interactions with applied auxins.

Destruction.—The existence of auxin-destroying systems in roots was early recognized [e.g. Gorter (69); Fiedler (61); Raalte (124)]. Later investigators [Tang & Bonner (147); Galston & Dalberg (63)] have shown that the roots may often contain the highest amounts of the IAA oxidase system, considerable amounts being present in etiolated shoots also, while it seems to be absent from, or inactivated by an inhibitor in, green leaves. For the properties of the enzyme system (or systems) the reader is referred to the reviews of Larsen (88) and Gordon (66). The products of the oxidation are still unknown. Studies with enzyme from etiolated pea epicotyls, which may be assumed identical with the enzyme of pea roots, indicate that indolealdehyde is probably only a minor product [Racusen (125)], the main products being as yet unidentified [Manning & Galston (102); Galston (62)].

Highly active extracts are easily prepared from roots of pea [Tang & Bonner (147); Galston & Dalberg (63); Åberg & Jönsson (8)], of kidney bean [*Phaseolus vulgaris*, Wagenknecht & Burris (157)], and of lentil [Pilet & Galston (120)]; low activities are shown by crude extracts of spinach (*Spinacia oleracea*) and oat roots (147), of pineapple roots [Gortner & Kent (71)], and of wheat and rye roots (8), while extracts of carrot roots (147), cress roots and flax roots (8) are virtually inactive. The existence of natural inhibitors, however, complicates the situation. A heat stable, noncompetitive inhibitor was shown to be present in pea seedling tissues, where its formation is promoted by light (147). Root sections of wheat and rye have been found to destroy IAA even more effectively than pea root sections, but the crude extracts had only a very low IAA oxidase activity (8). The most probable explanation is that inhibitory substances, which are *in vivo* separated from the IAA oxidase system, become liberated during the extraction. The wheat root extracts did in fact reduce the activity of a pea root extract strongly. In this case a considerable part of the inhibiting power of the extract is heat labile. No IAA oxidase activity has as yet been shown for

flax and cress roots, for which also the root sections are wholly inactive when placed in IAA solutions (8).

It seems very improbable that the IAA oxidase system of, for example, pea roots is as active *in vivo* as in crude extracts (8). On the whole virtually nothing is known with certainty about its physiological role [Gordon (66)]. Some function in the regulation of the native auxin level seems, however, likely [Galston & Dalberg (63); Pilet & Galston (120)], and a role in lignification has been suggested [see Galston (62)].

The Effects of Externally Applied Auxin on Root Growth

Intact roots.—According to the classical view of the auxin relations of roots the auxin content of intact roots is well over the optimum level, and normally only inhibitions of their longitudinal growth have been reported as the result of further auxin application: Nielsen (114), Cholodny (53), Boysen-Jensen (38), Kögl *et al.* (85), Jost & Reiss (79, 80), Meesters (104), Lane (87), Marmer (103), Thimann (151), Bonner & Koepfli (34), Swanson (145), Ready & Grant (126), Seiler (134), Audus (17), Åberg (1, 3, 4, 6), Åberg & Jönsson (8), Åberg & Khalil (9), Müller (108), Hansen (73), Pollock *et al.* (123). In several of these investigations, comprising corn, wheat, oat, flax, cress, pea, and lupine seedlings, extensive observations were made down to concentrations of the order 10^{-11} to 10^{-12} M of IAA or other auxins (3, 6, 9, 34, 80, 103, 123, 134). Under the circumstances it seems necessary to scrutinize closely the alleged cases of auxin stimulation of the roots of intact plants.

Firstly we have to consider the causes of error introduced by the biological variability. It seems to be a general assumption that a calculation of the statistical error attributable to the variation of the growth of individual roots within one experiment should be sufficient to test the "significance" of the results. Apparently such a method postulates the total absence of systematic errors which could influence the growth of whole groups of seedlings, e.g., those contained in a certain dish or tube or even in a group of dishes or tubes. The best way to test the existence of such errors is apparently the manifold repetition of the whole experiment, and the calculation of the variation of the results from a series of independent experiments. In the case of the flax root test this "interexperimental" variation has been found to exceed that expected from the variability of individual roots, and it seems fairly certain that this is at least partly due to a variation of the "sensitivity" of the roots to applied regulators, but that other causes may also be at work [Åberg (6)]. There may also be systematic errors inherent in the experimental design which may elude the investigator. Such errors can be detected by varying the technique slightly, or by repetition of the experiments by independent investigators. The reproducibility of a phenomenon under such circumstances must be the final criterion for its physiological significance.

Several reports on root growth stimulation by low auxin concentrations are apparently founded on single experiments without repetitions, and much confidence cannot be placed in the reproducibility of the results. The strong

stimulation of sunflower roots (*Helianthus annuus*) by 10^{-11} *M* IAA during a growth period of 5 hr. obtained by Naundorf (112) must thus certainly be repeated before acceptance. The amount of auxin delivered from root tips of this species has been found to be quite normal [Nagao (109); Faber (59)]. The stimulation claimed for *Allium fistulosum* roots [Borgström (35)] is not substantiated by quantitative data, and the stimulation reported by Levan & Lotfy (92) for *Allium cepa* roots growing in 10^{-11} to 10^{-12} *M* NAA solutions seems to be founded on too little material. For *Artemisia absinthium* seedlings Ashby (16) observed no stimulation after 4 hr., while stimulation probably lying outside the errors caused by the variation of individual roots occurred after 24 hr. for 5.7×10^{-10} *M* IAA and for a hundred times lower concentration of the corresponding aldehyde, but not for any concentration of NAA. The irregular type of the action curves suggests, however, that these "significant" stimulations might easily have disappeared upon repetition of the experiment several times and averaging the results.

For cress roots with an initial length of 5 mm. and grown for a period of 17 hr. on moist filter paper, Moewus (105) reported an optimal stimulation of about 15 per cent at application of 5.7×10^{-11} *M* IAA. The variation of the results from independent experiments with only 10 roots used for each concentration (105: Table 10) is incredibly small, which may partly be connected with the rejection of all experiments showing a control growth outside a narrow standard range. If, during some period, this range would accidentally deviate from the true average of the control growth, curious results might appear. A stimulation of the same strength has been refound by Clauss (55), while Linser (96) reports a stimulation of 10 per cent without giving any data on the variability. Ochs (115) obtained a maximal stimulation of 6 per cent, and Müller (108) was unable to find any statistically significant stimulation. Bentley (32) states that stimulations occur in the range 5.7×10^{-13} to 5.7×10^{-14} *M* IAA, but that they are not always obtained. Less variable results are said to be obtained with roots grown on agar, but the published data from such experiments (32: Table III) do certainly not indicate the existence of reproducible stimulation. The present author has studied the growth of cress seedling roots in solution culture (6), and the experiments have now been extended down to a nominal concentration of 10^{-16} *M* IAA, with a number of repetitions (*N*) up to 12, but no signs of a reproducible stimulation are apparent. The following values for the relative growth values (control = 100) with their standard errors may exemplify the situation: 10^{-11} *M* IAA: 100.4 ± 2.7 ($N=9$), 10^{-12} *M* IAA: 99.8 ± 1.7 ($N=12$), 10^{-14} *M* IAA: 101.8 ± 3.6 ($N=10$). On the other hand the cress roots used in these experiments are significantly stimulated by the antiauxin 1-NMSP both directly and when inhibited by externally applied IAA or 2,4-D.

The physiological significance of the occasional IAA-stimulation of young cress roots is not easily appreciated. It must be admitted that differences in the seed material used by different investigators may contribute to the different results obtained. Thus, Moewus (106) indicates that stimu-

lation may sometimes appear preferentially in roots longer than those originally recommended, depending on a later reduction of the IAA content to a suboptimal level. On the other hand, results obtained by this investigator in other fields have also turned out to be hard to reproduce [Ryan (132)]. That slight variations in the method used might be of importance is indicated by the results obtained by Ochs (115) for several different auxins, including IAA and 2,4-D. She modified Moewus' test by using 30 ml. instead of 5 ml. test solution, and found this to increase the stimulation from about 5 to about 15 per cent, but also to move it to 100 or 1000-fold lower concentrations. The most conspicuous stimulation was obtained with 4.5×10^{-15} *M* 2,4-D. The results are claimed to be statistically significant, but no comprehensive data on their variability are given. As the present author has been unable to find any reproducible stimulation of cress roots by 2,4-D concentrations down to nominally 10^{-16} *M*, he feels that it will be legitimate to await independent confirmation of Ochs' results before ascribing any general physiological significance to them.

The whole question of the presumed auxin stimulation of the growth of intact roots is further complicated by the possible existence of positive after-effects of auxin treatments. Thus Macht & Grumbein (101) reported slight stimulations (up to 20 per cent) for lupine (*Lupinus albus*) roots during the 24 hr. following a short treatment with dilute (about 10^{-9} *M*) auxin solutions. Sufficient data on the reproducibility of the results are, however, not given. Thimann & Lane (154) found the root growth of oat seedlings which had been treated with IAA (down to 7.4×10^{-9} *M*) and were then transferred to water, to be inhibited at first but to show conspicuous stimulation over the control growth later on. The roots of oat and wheat seedlings were also stimulated when growing on filter paper moistened with very dilute IAA solutions (about 10^{-8} to 10^{-11} *M*) for about a week. In this case the auxin may have disappeared during the first days, thus permitting the after-effects to outweigh an initial inhibition. Lundegårdh (98) found wheat roots to be initially inhibited by IAA in concentrations down to 10^{-10} *M* and lower. After long periods of growth the inhibition tended to disappear or even to change over into stimulation. Experiments with short auxin treatments (up to 4 min.) of corn roots, followed by observations on their growth in auxin-free solutions, were performed by Gast (64) who found a rapidly disappearing inhibition followed by stimulation over the control growth. In later experiments by similar methods this stimulation could, however, not be reproduced [Seiler (134)].

From the present point of view the stimulation reported by Pilet (117) for lentil roots might perhaps also be treated. Seedlings with roots of about 2, 17, or 42 mm. length were placed on moist sawdust and sprinkled over with IAA solutions of different strength. The growth of the roots was then measured daily for nine days or more. Even the longest roots which, according to Pilet, contain supraoptimal amounts of auxin, showed conspicuous stimulation, especially when treated with 10^{-11} *M* IAA. As the

roots are reported to contain auxin concentrations equivalent to 10^{-4} or even up to 10^{-3} *M* IAA in the growing zone the result is rather unexpected. Later experiments (119, 121) with young lentil seedlings placed in Petri dishes on filter paper moistened with the solution to be tested and with growth periods of four to six days, also show strong stimulation for young roots, but a conspicuous one even for roots with an initial length of 30 mm. when treated with 10^{-8} *M* IAA. Nor with this method is there a satisfactory contact between root and medium, or any certainty that the auxin has been present in the original amount during the whole of the experimental period. Stimulations of young roots were obtained also with low concentrations of maleic hydrazide (10^{-10} to 10^{-12} *M*), a substance which has never been suspected of having auxin properties.

Unilateral auxin applications on intact roots usually result in positive curvatures. Only such curvatures were found by Syre (146) who used root tips or agar blocks and lanolin paste with different amounts of IAA as auxin sources. Faber (59) obtained both positive and negative curvatures. The latter ones occurred after application of the most concentrated pastes, and they cannot be brought in line with the supposed stimulation of longitudinal root growth by low auxin concentrations. Their nature is not clear, but some relation to the root swellings occurring at high auxin concentrations does not seem improbable.

Here it should be mentioned that Lundegårdh (100) obtained strong stimulation of the growth of wheat roots by 10^{-3} *M* IAA, but also with comparable concentrations of hydrochloric acid. The stimulation occurred in the period 4 to 8 min. after the application, but disappeared in about an hour. The explanation of the acid effect as depending on a lowered dissociation of the auxin present in the root surface, thus bringing the amount of auxin anions to the optimum level, is interesting, but can apparently not be used for the stimulation caused by 10^{-3} *M* IAA.

Summarizing, it may be said that direct stimulation of the longitudinal growth of intact roots by low auxin concentrations has never been convincingly demonstrated as a regular and reproducible phenomenon. All investigators who followed the root growth with short intervals (1 hr. or less) have found inhibitions or unaltered growth immediately after the application of auxin in concentrations down to very low ones [Jost & Reiss (80); Gast (64); Seiler (134); Pollock *et al.* (123)]. The stimulation sometimes reported for infinitesimally low auxin concentrations mostly refer to tests using growth periods of 17 hr. or more. If real, it might be related to the positive aftereffects of auxin treatments which have been found by some investigators, and which could occur after the disappearance of the auxin from the medium. No hypothesis has been framed, however, which is able to explain either a direct or an indirect effect of external auxin concentrations thousands or millions of times lower than those probably present in the root tissues.

The experimental data bearing upon the existence of positive aftereffects of auxin treatments are not very extensive and, as they are partly

XUM

conflicting, further studies in this field are needed. Virtually nothing is known about the nature of these effects, but some connection with adaptation phenomena might be tentatively suggested. The question of an adaptation of roots to higher auxin concentrations was first raised by Lundegårdh (98), and the existence of such an adaptation, occurring after some days, has since been proven by Burström (46, 47). Ekdahl (58) found indications of a more rapid adaptation, but these results have not been confirmed in further experiments. A very slow weakening of the growth inhibition caused by IAA and NAA on isolated or intact *Pinus silvestris* roots was reported by Slankis (136). An adaptation to higher auxin levels might possibly result from changes in the receptor system active in growth regulation, or from an increased ability of the cells to destroy auxin. An adaptive increase in the IAA oxidase activity upon application of IAA, 2,4-D, NAA etc. has been reported by Galston & Dalberg (63), but too little is known about the role of this enzyme system in regulating the auxin concentration *in vivo* to permit a discussion of its possible significance in the present connection.

It should also be noted that negative aftereffects of IAA treatment have been reported for the growth of *Aegopodium* rhizomes and oat coleoptiles [Ball (26); Ball & Dyke (27); Bennet-Clark & Kefford (31)], and have been tentatively explained as attributable "to the conversion of IAA into an inhibitor, or to the production of an inhibitor from some other source as a result of the action of IAA" (26), a hypothesis which is supported by the absence of any corresponding aftereffects of 2,4-D treatment. If it is further assumed that the inhibitor is an antiauxin, the aftereffects of IAA upon shoot and root growth would be brought on a common basis. The accumulation of root growth stimulating substances in IAA solutions under the influence of oat roots [Bonner & Koepfli (34)] might possibly turn out to be of interest in this connection.

Isolated roots.—The delivery of auxin from glucose-fed root tips may go on for at least 17 hr. [Raalte (124)], and a continued synthesis in the tips of isolated roots may therefore be expected. Fiedler (61) grew isolated corn and pea roots in nutrient solution containing yeast extract (and hence probably IAA) for six to eight days. No auxin could then be obtained from the tips with the glucose-agar method. Chloroform extraction gave negative results even for corn roots from intact seedlings [cp. however, Boysen-Jensen (41)]. Broad bean roots on agar were found to lose the auxin in 19 hr., but to retain their ability to curve geotropically. These results must, however, be considered dubious. Auxin has since been found in roots grown in detached condition for fairly long periods [corn roots: Nagao (111), Segelitz (133), Würgler (160); pea roots: Nagao (111), Overbeek (116); sunflower roots: Nagao (110)], though the content may be lower than for intact roots (133, 116). The decrease is especially rapid for roots cultivated on plain agar (without sugar and nutrient salts), in which case the root growth also ceased within two days (111). After transfer to nutrient agar such roots soon began to grow and to produce auxin.

Several early investigations indicated stimulation of the growth of iso-

lated corn roots on synthetic media by low concentrations (about 10^{-10} to 10^{-12} *M*) of IAA or other auxins like NAA or 2,4-D [Geiger-Huber & Burlet (65); Fiedler (61); Würgler (160); Bein & Schopfer (28)]. The stimulation disappeared in the absence of sugar (160). No statistical data or repetitions of the experiments are, however, reported. From some data presented by Würgler (160) the dispersion of the growth of individual corn roots during four days can be calculated to 42 per cent of the average value, and Segelitz (133) was unable to find any significant stimulation by IAA (down to 5.7×10^{-12} *M*) because of the variation between individual roots. An impressive picture of the variability met with in such experiments is also given by the work of Tauja-Thielman & Pelece (148). Isolated corn roots were grown in solutions containing 2.86×10^{-15} to 2.86×10^{-5} *M* IAA. In some experiments the nutrient solution contained yeast extract and, hence, unknown extra amounts of IAA. The effects on the main axis length were quite irregular till the final inhibition set in at about 10^{-7} to 10^{-5} *M*. The most recent investigations on corn roots by Kandler *et al.* only gave a slight and statistically insignificant stimulation by 10^{-9} *M* IAA (10^{-10} to 10^{-12} *M* was without effect) (83), while no tendency to stimulation was found for 2,4-D (down to 10^{-11} *M*) or NAA (down to 10^{-12} *M*) (81).

Important observations on the nature of the IAA effects on isolated wheat roots have been made by Burström (46). A significant stimulation by 10^{-9} *M* IAA occurred in one out of two experiments. Even at this and lower concentrations (down to 10^{-11} *M*) the final length of the epidermis cells was initially decreased, though later on it increased up to and over the control value, a phenomenon presumably connected with a true adaptation of the cells [cp. also (47)]. For isolated barley (*Hordeum vulgare*) and oat roots Almestrand (11) failed to obtain any stimulation by IAA or NAA in the range 10^{-11} to 10^{-13} *M*.

For isolated rye roots, originating from some grains of a sample of the Petkus variety, Roberts & Street (129) found it possible, however, to stimulate the survival and growth of the main-axis meristems during repeated subculture by heated tryptophan solutions, containing an unknown transformation product (not IAA). The elongation of the main axis during a 14 day period was also slightly stimulated by 5.7×10^{-8} to 1.8×10^{-9} *M* IAA or 6.4×10^{-10} to 6.4×10^{-9} *M* 3-indoleacetonitrile. Some relation of these stimulations to the meristematic activity seems probable.

For isolated lupine roots (*L. albus*) Duhamet (56) reported a considerable stimulation of the elongation by 10^{-8} to 10^{-13} *M* IAA with a maximum at 10^{-11} *M*. The main part of the stimulation was related to an increased cell division. No data on the reproducibility of the results are given.

The growth of isolated pea roots has been reported to be slightly stimulated by 10^{-8} to 10^{-10} *M* IAA [Bonner & Koepfli (34)], while 2,4-D in concentrations down to 10^{-12} *M* was without effect [Bein & Schopfer (28)]. The stimulation by IAA was refound by Naylor & Rappaport (113) and observed to be optimal at about 5.7×10^{-9} *M*. Leopold & Guernsey (90) made growth tests over 24 hr. with 10 mm. long pea root tips, and found IAA to give

XUM

strong stimulation up to 10^{-7} *M*, sometimes even up to 10^{-5} *M*. Pea root tips (and root sections) have been found to show a peculiarly lowered sensitivity to IAA as compared to intact roots, a phenomenon which was tentatively explained as dependent on the action of IAA oxidase, activated at the cut surfaces [Åberg & Jönsson (8)]. The question then arises: is the stimulation found by Leopold & Guernsey and others really due to a direct effect of IAA, or is it perhaps caused by some transformation product of IAA? Adaptive responses may also be possible.

For isolated tomato roots no stimulations by IAA were found in the early studies [see (113)], and the wave-like activity curves with a minimum at 2.86×10^{-12} *M* reported by Tauja-Thielman & Pelece (148) only mediate an impression of a very high inter-experimental variation, particularly as the experiments were performed in nutrient solutions with yeast extract and, hence, unknown extra amounts of IAA. The absence of significant stimulation has been confirmed by Street *et al.* (142). The growth of the main axis is inhibited to 50 per cent at about 10^{-8} *M* IAA, and it does not deviate significantly from the control in the range 5.7×10^{-10} to 5.7×10^{-12} *M*. Very similar results were later obtained also with NAA (141). The inhibition caused by 3×10^{-8} *M* IAA was found to be correlated with a corresponding reduction of the final cell length (142), while high concentrations inhibited cell division also. To this may be added that the growth of the main axis is strongly stimulated by low concentrations (about 10^{-6} *M*) of antiauxins like 1-NMSP or 1-naphthoxyacetic acid, which also restore the growth of roots inhibited by NAA (140, 141). The interaction of 1-NMSP and externally added IAA was less conspicuous, though well comparable to that found for intact flax roots (140, 6).

For isolated *P. silvestris* roots Slankis (136) found very interesting morphological effects of IAA, NAA and other auxins. At sufficiently high concentrations a large number of dichotomously branched and swollen short roots appeared, which were morphologically very similar to the mycorrhizae found in nature. Because of increased branching, the total length of the root systems was strongly increased, especially in the range 3×10^{-9} to 3×10^{-8} *M* IAA or NAA, after three months culture in solutions which were renewed every fifteenth day. The growth of the individual axes is more difficult to judge from the presented data, but some stimulation may have occurred. If so, an influence on the meristematic activity of the root tips seems rather probable. A slow adaptation of the roots to higher auxin levels was observed.

Summarizing, it may be said that stimulation of the elongation of isolated roots by low auxin concentrations is by no means universal. In most cases the reproducibility of the alleged stimulations has been insufficiently studied. The often very long growth periods make effects conditioned by altered meristematic activity probable. Some indole compound may thus be necessary for the continued activity of rye root meristems (129). In one of the most closely analysed cases, however, the stimulation seems to be con-

nected with an adaptation of the roots to a higher IAA level (46). In most experiments the effects of such an adaptation might be enhanced by the disappearance of IAA during the growth period. On the whole, the normal reactions of isolated roots to applied regulators seem to be very similar to those of intact roots. When kept under proper culture conditions the isolated roots may presumably go on producing supraoptimal amounts of auxin, and like the intact roots they may be stimulated by the application of antiauxins.

Decapitated roots and root sections.—The obvious way to obtain roots with a very low auxin content is to lower the auxin production by decapitation or to isolate the zone of cell stretching in the form of root sections. A drawback inherent in such procedures is the activation of powerful IAA oxidase systems at the cut surfaces of many roots, which may cause complications especially when testing the response to IAA.

Even with such material growth inhibitions are naturally obtained if the applied auxin surpasses the optimum level, or if the auxin production in the subapical parts is efficient enough to bring the auxin level to a supraoptimum level. Positive curvatures of decapitated roots upon unilateral auxin application were reported by Hawker (74) for broad bean roots and by Snow (137) for corn roots. Also the longitudinal growth of decapitated roots is easily retarded by auxin, for example by the application of coleoptile or root tips [Cholodny (50, 52)] or of agar blocks with auxin [Boysen-Jensen (38)]. Jost & Reiss (80) obtained only growth inhibition for corn roots at immersion in 5.7×10^{-10} *M* IAA whether they were intact or decapitated.

Positive growth effects of IAA on decapitated broad bean roots were reported by Amlong (12, 13). Lateral application of 10^{-9} *M* IAA gave negative curvatures, and roots immersed in 10^{-8} *M* IAA grew at a speed twice higher than that of control roots during a period of 2 hr. Pilet (117) observed negative curvatures of decapitated lentil roots under the influence of the eccentrically replaced tips. Though indicating that the auxin concentration of the decapitated roots may sink under the optimum value, such results do not permit the conclusion that the same situation should be realized in the intact roots. Appreciable amounts of the auxin coming from the tip may have been destroyed at the cut surfaces by the action of the IAA oxidase system.

The most extensive data on the stimulation of root growth by auxin have been collected by Audus *et al.* (18, 19, 21, 22), using 2-mm. sections excised from the extending zone of pea seedling radicles. Stimulation by 5.7×10^{-11} *M* IAA, which may reach 50 per cent during the first 4 hr., has repeatedly been found, while the effect of 2,4-D in the corresponding concentration range (4.5×10^{-12} to 4.5×10^{-10} *M*) is negligible (21, 22). On the other hand, even substances like 1-NMSA, 1-NMSP, and 4-chloro-3-nitrobenzoic acid, all of them recognized antiauxins in the growth of intact roots, gave stimulation when applied in relatively high concentrations (about 5×10^{-5} *M*).

It should be mentioned that Åberg & Jönsson (8), using a corresponding

test, found the inhibiting effects of IAA to disappear in the range 10^{-7} to 10^{-9} *M* without being changed into significant stimulation. 10^{-5} *M* and higher concentrations of 1-NMSP gave inhibition, while 10^{-6} to 10^{-8} *M* did not significantly influence the growth. Such discrepancies might possibly result from minor differences in the technique used, giving rise to different activities of the IAA oxidase system at the cut surfaces or suchlike, but apparently they add considerable difficulties to the already complex situation.

Audus & Das (18) favour the explanation that the stimulations caused by IAA and, for example, 1-NMSA should be effected in identical ways in the same growth system. Such a hypothesis would gain a strong support if typical auxins other than the easily metabolized IAA could be shown to stimulate the growth of pea root sections. The absence of stimulation in the experiments with 2,4-D does instead detract from its applicability. If upheld, it becomes compatible with the assumption of a competitive antiauxin effect of 1-NMSA in many types of intact roots on adding the further hypothesis of a slight residual intrinsic auxin activity [cp. Åberg (7)] of the 1-NMSA molecules, which may become apparent in an exceedingly sensitive system practically devoid of native auxin.

Alternatively the possibility that the native auxin concentration of the pea root sections is still supraoptimal, must certainly be considered. Auxin production may, as earlier mentioned, go on in subapical root sections. The positive IAA effects found by Audus *et al.* would then possibly be related to the aftereffects discussed above, being perhaps connected with the metabolism of IAA.

The effects of regulators upon the growth of isolated root sections are apparently complex, and the explanations hitherto presented must be considered as tentative hypotheses only. As indicated by the interesting results obtained by Audus *et al.* they might, however, be able to throw new light on fundamental problems, and it is certainly imperative that they should be extended to a wider range of plants and growth regulators.

The nature of the root growth inhibition.—The auxin inhibition of longitudinal root growth is certainly not the result of a decreased intensity of the growth processes in general, but rather the outcome of a change in their course. This is clear already from the characteristic swellings of the subapical parts of inhibited roots, which were first described by Cholodny (53) and have since been studied by several authors, e.g., Jost & Reiss (80), Levan (91), Levan & Lotfy (92), Amlong (13), Burström (46), Kandler (81), Sun (143). The swellings originate from the cortical cells of the stretching zone which, instead of the normal polar extension, undergo an isodiametric or even a mainly radial enlargement. An excessive meristematic activity of the pericycle with the generation of numerous lateral root primordia may also contribute. The number of mitoses in roots treated with auxin concentrations strongly inhibiting to their longitudinal growth is not decreased during a period of several days (46, 91). Some of the mitoses may, however, become orientated in the transverse plane of the root, thus augmenting the

number of cell rows in the cortical parenchyma. Like the unusual type of the following extension growth this phenomenon indicates a change in the polarity of the cells, which may well be a basic phenomenon in the auxin inhibition of longitudinal root growth. Consistent with such a view are the early observations of Amlong (13) that the plastic extensibility of root cells is still increased by auxin concentrations which strongly retard the longitudinal growth, and drops below the control value only at concentrations which must be deemed strongly toxic (10^{-2} *M* IAA). Adequate data on the quantitative relations between longitudinal growth inhibition and root swelling are still very scanty. It may be stated, however, that the swelling does usually not compensate for the longitudinal growth inhibition volumetrically. On the other hand, such a volumetric compensation might not necessarily be expected, the change in growth being qualitative in nature rather than quantitative. It should be mentioned that subterminal root swellings similar to those caused by auxin may be caused also by other substances tending to destroy the polarity of the cells, e.g., coumarin [Pollock *et al.* (123)].

From studies on isolated roots as well as roots of intact wheat plants which were either inhibited by IAA or stimulated by antiauxins, Burström (46, 48) concluded "that the profound changes of the growth behaviour leave the synthetic processes apparently unaffected." Further studies on isolated corn roots by Kandler *et al.* (81, 82, 83) have shown that the production of dry matter may even be increased by auxin concentrations which inhibit the growth in length. As the nitrogen content was little influenced this increase is probably due to a more rapid carbohydrate assimilation. An antiauxin [α-(4-chlorophenoxy)-isobutyric acid] which strongly increases the longitudinal growth, had the opposite effect. High auxin concentrations (10^{-4} *M* IAA, 10^{-5} *M* 2,4-D or NAA), which may be assumed to exert a nonspecific toxicity, caused a depression of dry matter production and nitrogen assimilation.

As observed already by Cholodny (53) the auxin swellings of roots become densely covered with root hairs, which may reach an excessive length [Burström (46); Ekdahl (58)]. The growth rate of the root hairs has been shown to be practically uninfluenced by auxin concentrations (10^{-6} to 10^{-5} *M* IAA) which cause a maximal inhibition of the longitudinal root growth [Meesters (104); Lundegårdh (99); Ekdahl (58)]. Apparently, such data are a further strong support for the interpretation of auxin inhibition of the longitudinal root growth as a change in the type of growth rather than a general depression of the growth functions. Isolated *P. silvestris* roots represent a special case in that they become more densely covered with root hairs at applications of IAA in concentrations up to 3×10^{-7} *M*, but develop swollen parts free from hairs in still higher concentrations [Slankis (136)].

Antiauxin Effects

The antiauxin concept relates to the growth of both shoots and roots, and the full characterization of a growth regulator as an antiauxin should

include its effects on both types of organs. The most probable hypothesis on the main mechanism of antiauxin effects assumes a binding of the auxins to some receptor of protein nature [cp. Åberg (7)]. Only when bound to these active sites, probably by many weak bonds ("multipoint attachment"), do the auxin molecules exert their growth-regulating effects. A typical competitive auxin antagonist would then be a substance which is bound to the auxin receptor in the same place as the auxins, but which gives a complex unable to initiate the usual growth responses. Under the circumstances the growth of shoot parts with suboptimum auxin levels must be retarded by application of an antiauxin which competes with the native auxin for the active sites. In roots, with supraoptimum auxin levels, the same competition must lead to an accelerated growth in length.

Many substances are now known (7) which follow this scheme. (*a*) They counteract the inhibiting effects of externally applied auxin upon flax root growth, even if applied in concentrations which are without effect on or slightly depress the growth of control roots. (*b*) They stimulate the growth of intact flax roots slightly and that of wheat roots strongly when applied in proper concentrations. The latter ones presumably contain a more highly supraoptimal content of native auxin than do flax roots [cp. (6)]. (*c*) They inhibit the growth of oat coleoptile sections. (*d*) As far as tested they also counteract the effect of externally applied auxins on oat coleoptile sections in the manner expected. Other regulators show deviations from the pattern mentioned, e.g., no stimulation of flax roots, but this is only natural with respect to the possible occurrence of various complications (competition during uptake and transport, synergism, effects on auxin metabolism, species and organ differences in the receptor system, nonspecific toxicity, and so on).

It is true that root elongation is a complex phenomenon, but it is none the less true that it is a well integrated process which may be regulated as a whole. The complications have recently been exhaustively discussed by Torrey (156), and the question shall not be reopened here. If it is important not to underestimate the difficulties, it is, however, equally important not to exaggerate them. For a short time test it is obvious that cell elongation must be the main factor in determining root growth so long as the meristematic cells are not used up. Later on a considerable retardation depending to a large extent on a shortage of meristematic cells might be visualized [e.g., inhibition by certain concentrations of maleic hydrazide, cp. Åberg (3)], or an apparently unchanged growth rate might possibly result from fewer cells elongating to a greater final length. Hansen (73), studying the effects of a large number of auxins and antiauxins on the growth of wheat roots during three days, found, however, that this is normally not the case. Antiauxin applications in nontoxic concentrations not only increased the final cell length; they also augmented the number of elongated cells, while the reverse was true for the auxins.

Other sources of error in interpreting the antiauxin-auxin interactions might result from the complex nature of the cell elongation process and the factors initiating it or causing it to stop. The relations of auxins and antiauxins to supposed phases in the elongation process are, however, unclear [Burström (49), cp. Torrey (156)], and little apt to give a coherent picture from the available growth data. Hence, the hypothesis of competitive antagonism as the fundamental basis of the phenomena indicated above (*a* to *d*) seems to be the only feasible one at present. Apparently, effects on auxin uptake, transport and metabolism, together with possible effects on other metabolic processes and nonspecific toxic effects occurring at high regulator concentrations [cp. Åberg (7); Bennet-Clark (29); Kandler (81)] must be able to cause deviations from a simple scheme, and much work may still be needed for their clarification.

If the possibility of a competitive inhibition and reversal of the auxin effects is accepted, the next step is to inquire into the existence of substances intermediate between the typical auxins and typical antiauxins [Åberg (2, 5, 7)]. The molecules of such a substance should have a conspicuously decreased, though not totally eliminated, activity in initiating the auxin responses when bound at the proper sites, or, in other words, their "intrinsic auxin activity" should be lowered. The effects of "intermediate" regulators would, like the effects of auxins and antiauxins, be a function of their intrinsic auxin activity and their "affinity" for the active sites. In systems saturated with an auxin of high intrinsic activity they should cause antiauxin effects, while their positive auxin effects should appear when applied to systems previously poor in auxin. The concentration-response curves of substances presumably belonging to this class are normally of a complex type, an initial inhibition of oat coleoptile section growth being followed by a reversal or even by strong stimulation at higher concentrations, while wheat roots show the inverse type of response [for examples, see (7)]. Much more work is needed to clarify these interesting phenomena, which are perhaps fit to be used as a touchstone for different hypotheses on auxin and antiauxin actions. For their quantitative elucidation it may be necessary to resort to the assumption of active sites of similar but not identical types as was newly done by Thimann (153) in his effort to interpret synergistic phenomena. As far as a physicochemical action on protein molecules or the like is held responsible for auxin effects, such an additional hypothesis seems rather natural. The same is valid also in respect to probable species differences in the receptor systems [Åberg (7); Audus & Das (18)]. The latter authors even visualize that the receptor proteins might vary from one organ to another in the same plant. A further possibility which might perhaps be worthy of consideration, is the existence of regulator effects of different signs. An antiauxin would then possibly act both by competition with auxin at the growth centres and by exerting an effect opposite to that of auxin when bound to these centres.

XUM

Auxins and Root Tropisms

Geotropism.—The Cholodny-Went theory of geotropism is only compatible with the assumption of a supraoptimal auxin content of normal roots, and, as we have tried to show above, no conclusive arguments against such a view seem to have been set forth. Good arguments against the necessity of statolith starch for the geotropic response of roots exist [Syre (146)], and Pilet's attempt to revive the statolith hypothesis seems to be of little success [Brauner (43); Larsen (89)].

It was early found that certain aromatic substances like erythrosin could completely eliminate the geotropic responses of roots [see (39, 146)], and later the number of such substances has been largely augmented [Jones, Metcalfe & Sexton (78)]. Most of them are benzoic acids with large *ortho* substituents (e.g., erythrosin, *N*-naphthylphthalamic acid, 2-benzoylbenzoic acid), but other types also exist. Boysen-Jensen (39) concluded from experiments on the auxin delivery to glucose agar that erythrosin drastically lowers the auxin content of broad bean roots, but this was made dubious by Syre (146) who found equal auxin amounts at chloroform extraction of normal and erythrosin-treated roots. The longitudinal auxin transport was completely inhibited in corn roots, but little influenced in broad bean roots. Jones *et al.* (78) tentatively suggest that the antigeotropic substances act through a specific interference with the lateral transport of IAA. The possibility of effects on other parts of the auxin mechanism must certainly be left open, however. Ageotropism has been induced in timothy roots (*Phleum pratense*) by treatment with 2,4,6-trichlorophenoxyacetic acid [Brumfield (45)], which is known to exert antiauxin effects in several types of processes [see (7)]. Rufelt (130, 131) has studied the influence of α-(4-chlorophenoxy)-isobutyric acid and of α-indoleisobutyric acid upon the geotropism of flax and wheat roots. Both of these substances certainly have a conspicuous antiauxin activity component, but differ in some respects. Their effects on the geotropic reactions are not identical, the indole but not the phenoxy compound being able to change the normal positive response into a negative one. Also auxin applications naturally influence the geotropic curvatures of roots. IAA has thus been shown to prolong both presentation and reaction times of flax roots (130). The reaction times of wheat and timothy roots are also increased (45,130).

The analysis of the effects of growth regulators upon the geotropic reactions must necessarily meet with great difficulties. To the complex phenomenon of longitudinal growth comes the lateral polarization of the organ under the influence of gravity and the lateral transport of auxin, which both are little known processes and which both may possibly be influenced in different ways by applied growth regulators. If substances really exist which specifically influence the lateral transport of auxin in geotropism (78), they must certainly become valuable tools in the further study of this phenomenon.

Phototropism.—Pilet's (118) views on the role of auxin in the photo-

tropism of lentil roots in ultraviolet and visible radiation have already been fully discussed by Brauner (43). Interesting new data, showing the complicated nature of the root curvatures induced by ultraviolet radiation, have been adduced by Brumfield (45). Using timothy roots he found a first positive phase, reaching its maximum about 40 min. after exposure. Later it was changed to a negative curvature, occurring somewhat nearer the root tip than the positive one and reaching its maximum about 100 min. after exposure. Both phases were completely inhibited by a concentration of 2,4,6-trichlorophenoxyacetic acid which but little influenced the growth rate. Such results are hardly compatible with a simple hypothesis of photochemical auxin inactivation as the cause of phototropic root curvatures, and instead point to some connection with the mechanisms controlling the geotropic response.

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THE HISTORY AND PHYSIOLOGICAL ACTION OF THE GIBBERELLINS[1]

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During the past thirty years a large number of Japanese botanists have been working on a strikingly potent and unusual group of plant growth substances, attracting little attention in the western world. These substances were first shown to be produced by a rice disease fungus, *Gibberella fujikuroi*, by Kurosawa, a Japanese plant pathologist working in Formosa. They were named gibberellins after the fungus by a group at the University of Tokyo, notably including Yabuta, Sumiki, and Hayashi, who are responsible for their isolation and the first studies of their properties. About five years ago, their work came to the attention, apparently independently, of a group at Imperial Chemical Industries in Britain, and another group at the United States Department of Agriculture. These two groups confirmed the Japanese work and contributed their own findings, bringing their results to a much wider audience.

It is the intent of this review to summarize this development, placing emphasis on the Japanese literature which is not readily available to western readers. Such topics as the disease, the fungus, and the chemistry and production of the active substances will be covered only so far as seems pertinent to a more complete description of the action of the gibberellins on plants.

The Fungus and Disease

Ito & Kimura (57) attribute the earliest known description of what is now called the bakanae disease to Konishi (71), a semiliterate farmer who dictated an agricultural book in 1809 [see also Bokura (6)]. His explanation, not published until 1828, of the disease was based on the "in-yō" philosophy, in which responses are interpreted as resulting from disturbance of a balance of positive and negative factors.

The most characteristic symptom of the disease is the appearance of tall thin plants, markedly overgrowing their uninfected neighbors. Thus the name "bakanae" (foolish seedling), which is now generally in use, but many other colloquial names are known. Affected plants are not only taller, but the internodes and leaf sheaths are longer, the leaves longer, narrower, and thinner, and the angle the leaves form with the culm increased. Root growth and tillering is reduced, and the plants usually appear chlorotic. Mild symptoms are reminiscent of etiolation, and the overgrowth could easily be dis-

[1] Abbreviations used: IAA, indoleacetic acid; I. C. I., Imperial Chemical Industries; U.S.D.A., U. S. Department of Agriculture; other abbreviations introduced in footnote 7.

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missed as the result of a mixture of two varieties of rice. In light cases flowering may be two to three days earlier, but the ears are smaller and the yield is reduced. More severe infections lead to adventitious roots forming at the aerial nodes, the stem curving at the nodes, leaf curl, foot rot, and death before flowering. Often there is no external evidence of the fungus until the disease is well advanced.

The first descriptions of the disease with its causal fungus are attributable to Hori (51, 52, 53).[3] There are other detailed Japanese descriptions (74, 75, 100, 120, 121, 124, 125, 153). The most complete monographs are those of Ito & Kimura (57) which cover aspects of the history, symptoms, methods of infection, treatment, and recommended agricultural practices to reduce its incidence.

The disease has since been described in India (160), in the Philippines (116), in China (17), in British Guiana, where it is known as "man" rice (93), in Italy (2, 16, 27), and in Ceylon, where it is called "wanda peedema" (sterile ripening) (112). An English review of some aspects of the disease is available [Padwick (111)].

Hori (51) identified the agent of the disease as an imperfect fungus, *Fusarium heterosporum* Nees. Later, Fujikuro discovered the perfect stage and this was described as *Lisea fujikuroi* by Sawada (119). Sawada was uncertain of Hori's identification of the causal fungus, and suggested that Hori had instead described the carrier of "akakabi," a disease which occurs during the flowering period. However, Sawada with Kurosawa (121) substantiated his identification by inoculation and reisolation experiments, which were repeated by Hemmi & Seto (45) and Seto (124, 125). Noting the similarities of this microorganism to *Gibberella moniliformis* Winel., Ito (see 57) suggested *G. fujikuroi* as a more appropriate generic designation. Disputing this assertion, Kurosawa (78) examined 104 fungus strains collected from bakanae seedlings and showed that all but one caused the overgrowth symptom, while twelve fungi identified as species of *Gibberella* produced either inhibition or no effect. Only one of these twelve had been collected from rice. He concluded *G. moniliformis* could not be the bakanae fungus and defended the retention of the genus *Lisea* for the organism. A further complication ensued when Kurosawa (76) found that his bakanae strains would cause the "akakabi" disease which Sawada had previously ascribed to Hori's *F. heterosporum*. This latter problem was solved by Hemmi *et al.* (46, 48) and Seto (132, 134) who demonstrated that when seeds were infected with the fungus, the bakanae effect resulted, but infection of mature plants during flowering led to the "akakabi" symptoms (appearance of red fungus spots on the rice husk). The two organisms were thus the same.

When Wollenweber revised the *Fusaria* (173), he agreed with Ito's suggestion and transferred the name of the perfect form as *G. fujikuroi* (Saw.) Wr., this having priority over *G. moniliformis* Winel. which had been first

[3] An 1877 Italian report (15) cited by Baldacci (3) lists a possibly identical fungus found on rice, but no disease symptoms are described.

used to describe the same fungus occurring on maize. The imperfect form was designated *F. moniliforme* Sheld., from the first valid description of the fungus on maize (135), its identification as *F. heterosporum* by Hori (51) being apparently based on an incomplete description. Thus the bakanae fungus was tied taxonomically to a microorganism, which as Kurosawa (78) had shown, failed to cause the bakanae effect. Seto (124, 125) provided a clue to this anomaly when he demonstrated that certain strains of the bakanae fungus failed to produce the overgrowth symptoms, and suggested (124) that two factors, one inhibiting and one promoting growth, might be responsible. Nisikado (100) examined the morphological criteria and agreed with Wollenweber's conclusion that the bakanae fungus was the same *F. moniliforme* which was known to attack many plants. Using six strains isolated from rice, maize, and sugar cane, he obtained overgrowth of both maize and rice from the strains isolated from rice, and also from one strain isolated from sugar cane. In addition, Nisikado & Matsumoto (101) showed that the effective strains could cause overgrowth symptoms in sorghum, sugar cane, millet, barley, and (less so) in wheat, as well as in rice and maize. Later results (102) showed a gradation of growth in infected plants from 200 to 86 per cent of controls caused by 72 strains of the same morphologically defined fungus, and in which individual strains exhibited significant variation from test to test. Comparison of the Japanese fungus with the Indian fungus described by Thomas (160) led to the conclusion that the fungi varied within the same limits and were the same (104). Seto (127, 128, 131) has shown that a large proportion of nonbakanae plants in infested fields contain the bakanae fungus. Hemmi (47) was able to isolate the fungus from 97 per cent of bakanae seedlings, from 63 per cent of stunted seedlings, and from 32 per cent of apparently normal seedlings. Hence, variation seems to occur in the field.

This variation of *Fusaria* has been noticed by many observers. Leonian (85), for example, showed that even single spore cultures changed characteristics constantly. Taxonomic revisions have been suggested for this reason (32, 143). Recently, Zachariah *et al.* (200) have shown that normal development of *F. moniliforme* required fluctuations of light and temperature, and this suggests the entire problem of classification may have to be re-examined. The names for the bakanae fungus now generally used are *Fusarium moniliforme* (imperfect stage) and *G. fujikuroi* (perfect stage); the latter is only found occasionally.

F. moniliforme is known to attack a wide variety of hosts, among the most important being maize, cotton, rice, and sugar cane. It has also been reported from an insect (110), on wool (169), and has been artificially inoculated onto a mammalian host (60). Maps of its distribution are available (18). Histological studies of the course of infection in rice (27, 57, 106), maize (166) and sugar cane (24) have been carried out. The spores are exceptionally long-lived (57, 105, 121). Its pectinases have been studied (141). A large number of papers contain information on its morphological and cul-

tural characteristics (2, 13, 17, 24, 25, 27, 32, 57, 77, 100, 103, 104, 110, 119, 120, 121, 148, 153, 156, 163, 165). Because of its variability, identifications of the organism must always be treated with some reserve.

The agreement on the identity of the bakanae organism has left one important question unexplained. Why does a broadly distributed fungus apparently cause its overgrowth effect naturally only in rice? No natural occurrence of an overgrowth in plants other than rice seems to have been reported, although artificial infections have led to overgrowth in maize (2, 26, 36, 100, 101, 104, 107), barley (2, 101), sugar cane (101), sorghum (101), millet (101), wheat (101, 107, 142?) and oats (36). Both Nisikado (100) and Baldacci (2) found maize more responsive than rice. A conclusive answer to this paradox has not yet been given, but the following clues are available.

Ito & Kimura (57), Shimada (138, 139) and later Stoll (148) reported that once-effective strains maintained in artificial culture lost their bakanae-inducing ability after a few months, and this could not be re-established by inoculation on rice and reisolation. However, Stoll did restore the overgrowth symptom when he grew the fungus on a rice grain medium. It is thus possible that some nutritional factor supplied by rice promotes the overgrowth.

More evidence exists for an effect of temperature on the infected plant. Many workers have shown that the optimum temperature for the growth of the fungus is about 27° C. But for the infection 30°C. is optimal (76, 157, 158), whereas Seto (126, 128, 130) stated that 35°C. is best for the bakanae effect. Although Seto (132) and Hemmi (49) have indicated that different strains were not identical in temperature response, it seems clear that the optimum for overgrowth of the plant is about 5°C. above that which is optimum for the *in vitro* culture of the fungus. De Haan (36) found that *F. moniliforme* caused significant though not striking overgrowth in barley and maize at temperatures above 27°C., while rice and oats showed some bakanae symptoms at most temperatures. His data indicate that *Fusarium herbarum* also caused strong overgrowth in maize at elevated temperatures, and *F. culmorum* in rice. Much later, Nisikado & Nakayama (107) reported 31°, 31° and 28°C. as optimal for the overgrowth of infected rice, maize, and wheat, respectively. Baldacci (2, 3) found 35°C. optimal for rice and ascribed the rarity of the disease in Italy to the generally lower temperatures prevailing there. Peiris (112) has noted that the disease in Ceylon is more prevalent in the warm season crop of rice; also noted in Formosa (74, 122). Hashioka (37) found temperate varieties of rice to show overgrowth less frequently than tropical ones. However, the temperature effect fails to explain why overgrowth has not been reported in naturally infected maize and sugar cane.

Another variable is the soil moisture. Sawada & Kurosawa (121) and Takahashi (154) reported less disease in well watered beds, but Takahashi (157) also found that the greatest overgrowth occurred at the same water

level which was optimal for the growth of the variety of rice. Seto (129) stated that in a dry bed the plants are more apt to be stunted and in a wet bed to show the bakanae symptoms; he may have had an exceptionally virulent strain. Thus there is an effect of soil moisture, but it may vary with the rice variety and perhaps the fungal strain. It may be that the bakanae effect is most pronounced under growth conditions optimal for the rice variety used.

Borrow *et al.* (13) have commented on the rarity of bakanae induction by strains of *F. moniliforme* not isolated from rice, citing Nisikado's (100) results as the only exception. This is supported by the extensive experiments of Leonian (86), Ullstrup (163), and Voorhees (165), which should have detected any growth-promoting activity of their strains of *F. moniliforme* on maize. And van Dillewijn (24) noted that when sugar cane is attacked at the top of the shoot by *F. moniliforme*, characteristically the lateral buds do not grow out, as would be expected if gibberellin were produced. However, Edwards detected (25) promotion of seed germination in infected ears of corn, and also (26) found that *G. fujikuroi* promoted growth of corn. Significantly, this was only true at higher temperatures. Edwards ascribed his results to antagonism of *G. fujikuroi* to *Trichoderma viride*, which had infected all his plants, including his controls. He could not show this antagonism in culture. It seems then, that his report, as well as that of de Haan (36) should be counted as overgrowth produced by nonrice strains of *Fusaria*.

Many other fungi are known to influence plant growth, and it now becomes of greater interest than ever to examine them. Baldacci (4) and Brian (8) have compiled lists of fungi which cause relatively specific growth effects in plants and which should provide a starting point for further examination of the action of fungal metabolites.

The Production of Gibberellins

All research on the gibberellins stems from the work of Kurosawa (73). In 1926, after several failures, he succeeded in producing the bakanae effect in rice and maize seedlings solely by treating them with a culture medium in which *G. fujikuroi* had been grown. The treated plants were elongated, slender, paler than normal and the root growth was inhibited. The appearance of the shoot was not caused by interference with root growth, since derooted plants behaved similarly and the effect could also be caused by injecting the culture medium into the hollow of the stem. The substance responsible was nonenzymatic since it survived three hours boiling. Culture media from other fungi (not specified) failed to cause the same effect, and were all inhibitory to growth. Kurosawa tested bakanae-resistant rice strains with his extract, and suggested that their response to the culture medium paralleled their resistance. Although not clearly indicated by his data, this was later shown more convincingly by Yoshii (199). He also tried unsuccessfully to adapt rice seedlings to the active substance by gradually increasing the

concentration over several days. Hemmi & Seto (45) and Seto (124, 125, 127) at the University of Kyoto confirmed Kurosawa's report of the active filtrate and also could not duplicate the effect with other fungi.

The properties of the active principle were soon examined by several investigators. Ito & Shimada (56) at Hokkaido University confirmed the heat stability of the substance but found that its activity disappeared on ashing; it was not volatile, could be dialyzed, and was adsorbed on animal charcoal (see also 156). Other *Fusaria* failed to produce the overgrowth substance. Kurosawa (80) substantiated and amplified these observations by showing that it was stable to autoclaving, freezing, sunlight, and short exposures to ultraviolet light. He apparently was in error in reporting insolubility in a number of organic solvents, since Shimada (136, 138) found that the active substance was soluble in ethanol, acetone, chloroform, ether, and water. From a chemist's viewpoint, the solubility is low in the last two solvents (19), though amply sufficient for bioassays. Furthermore, the active principle could be eluted after adsorption on charcoal by acetone, alcohol, or ether, but not by water, and the eluate could cause the bakanae effect in wheat, barley, maize, Azuki, and soya beans, as well as rice (138). Although the active principle is heat-stable, activity of filtrates is generally less on boiling (56, 124, 155, 177) and it is possible either that part of the activity is heat labile or that an inhibitor is produced on heating.

This work was paralleled by studies of the optimum conditions for production of the material. Seto (125, 127) concluded that 0.2 to 0.1 per cent glucose was optimal for production of the active factor, though giving only poor growth of the fungus. Takahashi (154, 155) at Mie Agricultural School also found that although fungal growth was optimal at 20 per cent glucose in Knop's solution, optimal production of the overgrowth substance was at 0.5 per cent. His conclusion was supported by dilution experiments designed to check if inhibitory substances were masking the overgrowth response at high glucose concentrations. Studies of the fungus have found it capable of growth on other carbon sources (2, 13, 54, 58, 66, 80, 140, 147) including even cellulose (133) and wool (169).

As to salts, Kurosawa (79), in a study of 90 mixtures, found that $MgSO_4$ always led to the formation of a growth-inhibitory filtrate. Later experiments (80) he interpreted as indicating that only NH_4Cl and KH_2PO_4 would meet the requirements of the microorganism, and that these produced the most active filtrate. This is not a complete medium and even at the time Ito & Shimada (56) stated that $MgSO_4$ was required. Nonetheless, Kurosawa's results were accepted by Yabuta *et al.* (196) who used his medium successfully for many years. They apparently had complete media available for comparison, since they specified one for the production of a growth-inhibitory substance (180). Borrow *et al.* (13) were unable to achieve satisfactory results with Kurosawa's medium, and it seems likely that both Kurosawa and the Tokyo workers were unknowingly introducing small amounts of magnesium and sulfur from some unexpected source. Recent

workers have simply added $MgSO_4$ (147), or used Czapek-Dox (13), Raulin-Thom (13), and modified Raulin (66) salt formulations. Other nitrogen sources were examined by Shimada (140) who found $(NH_4)_2SO_4$, asparagine, and peptone preferable to $NaNO_3$ and specified acid pH's.

Among trace elements, boron seems to be important for the fungus and manganese and zinc less so (117, 198). The I.C.I. patent application (202) specifies Fe, Cu, Zn, Mn, and MoO_4. Some heavy metals may be inhibitory since a number of tank-lining steels, including stainless steel, reduced the production of the physiologically active factor (67). Vitamins are not required (28), but a rice embryo extract improved growth on an otherwise synthetic medium (58).

In all these experiments it was difficult to make valid comparisons because of the variability of the bioassays used. Rice seedling tests are satisfactory in a qualitative sense, but because of variation in seeds, environmental factors, and perhaps most important here, the presence of growth inhibitors in the fungal medium, they can yield at best only semiquantitative results.

Upon the entry of the University of Tokyo group into the field (174, 196), a reliable rice seedling bioassay was developed, with dilutions designed to minimize inhibitor effects. With this they explored the influences of pH, glucose and glycerol concentrations, length of culture, and fungus strain. This extensive work confirmed the earlier reports that the active substance was best produced at relatively low carbon source concentrations of 1 to 3 per cent, 1.5 per cent glycerol being optimal and better than glucose. The initial pH range of 3.0 to 4.4 gave the most active assays, 3.4 being recommended. Best results were obtained with 30 to 40 day cultures. It should be noted that the British workers now recommend (202) much higher carbon source concentrations.

The effect of temperature on activity of filtrates has only been detailed by Kurosawa (79), who found 20°C. best. Yabuta *et al.* (191) incidentally list some room temperature limits, and the British workers (202) state 25° to 33°C. can be used. Since the bakanae effect itself is most pronounced at relatively high temperatures, and whereas nearly all fermentations use 25°C. or less, further examination of this variable might be worthwhile.

Neither does aeration seem to have been carefully studied. *G. fujikuroi* grows very well anaerobically, and aeration only slightly increases growth while inhibiting conidial production (50, 54, 118). However, Kitamura *et al.* (67) found that shaking reduced the time required to attain maximum biological activity, and aeration by pressure still more. They obtained low yields, estimated as 8 mg. active substance per liter, whereas still cultures averaged 37 mg./l. Stodola and co-workers (147) using short term, aerated cultures obtained about 20 mg./l. Borrow and collaborators (13) in surface cultures obtained 40 mg./l., and in aerated submerged cultures yields approaching 200 mg./l. were obtained in 18 days. The patent application (202) specifies stirred cultures and cites yields as high as 544 mg./l. Length of time

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in culture probably explains these differences better than aeration methods used, and the British workers have emphasized (13, 202) that the major part of the active material is produced after the growth of the mycelium has stopped. Thus Kitamura *et al.* (67) noted that pH, NH_4 nitrogen and glycerol all decreased with time roughly inversely proportional to mycelial formation, while maximum biological activity was always reached when mycelial growth had stopped. Apparently optimum yields are obtained when the mycelial growth is checked by exhaustion of nitrogen, and an excess of carbon source is still present (202). The British workers believe (13) that the active factor is produced metabolically and is not a result of autolysis. Analyses of fungus mycelium components have been carried out (185).

The University of Tokyo group (180) isolated an inhibitory material from the fungal extracts, thus confirming the frequent suggestion that both a growth-stimulatory and a growth-inhibitory material were produced. This inhibitor was named fusaric[4] acid and was shown to be 5-*n*-butylpicolinic acid. This compound is a strikingly potent growth inhibitor and wilting agent, being effective in some plants in concentrations as low as 0.1 mg./l. Recent studies in Japan (159) and in Switzerland (31) have shown that its effectiveness seems to be due to its chelating properties, which make it a strong inhibitor of heme-containing enzymes. Its action can in part be reversed by addition of minor elements. Fusaric acid has been shown to be produced by several other fungi (31). Its detection by paper chromatography has been described by Zähner (201). Stoll (148) found indications of another, probably proteinaceous, inhibitor in *F. moniliforme* extracts, and also isolated dehydrofusaric acid. This had biological properties similar to fusaric acid.

The growth inhibition problem having been clarified, the University of Tokyo group now methodically reinvestigated the properties of the bakanae-inducing substance (175, 176, 177). Animal charcoal was confirmed as the best adsorbent, and the active factor, named gibberellin by Yabuta (174), was best eluted from it by basic methanol. With these facts in mind they devised the general scheme of isolation (177) which has been used with minor modifications by all workers since. The culture filtrate is treated with active carbon, the adsorbed substances eluted with methanol-ammonia, the methanol extract evaporated, the residue taken up in bicarbonate, ether extracted, the aqueous layer acidified, and the gibberellins taken up into ether. The last steps have varied from time to time; some papers included lead acetate precipitation, ethyl acetate (13, 67, 147, 202) or butyl acetate (152) in place of ether, and countercurrent distribution has been used as a final step (147, 152).

As noted above, the yields of gibberellin have varied from 8 to 544 mg./l. Stodola (145) has concluded that commercial production should now present no problem.

[4] Fusaric acid is the name used by its discoverers (179, 180). Recently, the term fusarinic acid for apparently the same substance has frequently occurred. This misnomer apparently resulted by retranslation from the German "fusarinsäure."

The Chemistry of the Gibberellins

After these years of preliminary work on purification of the bakanae factor Yabuta & Sumiki announced in 1938 (181) the isolation of two crystalline, biologically active principles, which they named gibberellins A and B.[5] In a series of papers on their degradative chemistry (186, 187, 188, 183, 197, 123, 152, 65—in order of appearance) it was shown, among many other points, that A is converted by heating (50° to 70°C.) in dilute acid into gibberellin B. But raising the temperature to boiling converted both A and B into another compound, gibberic acid, which is not biologically active (187). A third biologically effective compound, gibberellin C, was also isolated from acid treatment of gibberellin A (187, 65, 152). These studies established the nature of the gibberellin skeleton by showing that gibberene, obtained by selenium dehydrogenation of gibberellins A, B, or gibberic acid, was a fluorene derivative (188). It was nearly ten years before western abstracts of these papers became available.

The first work on the gibberellins in the United States was carried out by J. E. Mitchell & Angel (95) at Camp Detrick, Maryland. They cultured the fungus and obtained the overgrowth of beans using the fungal culture medium. Their work prompted a group at the U. S. Department of Agriculture, headed by Stodola (147), to undertake the purification of the active principle. Apparently almost simultaneously, workers at Imperial Chemical Industries in Britain also began large scale preparation of the material [Borrow *et al.* (13)].

Both the American and British work resulted in the isolation of an entirely new compound from their fermentations. Named gibberellin X by the U.S.D.A. team (147), and gibberellic acid by the I.C.I. group (22), the latter name has now been accepted by all involved. Gibberellic acid clearly differs from the gibberellin A of the Tokyo workers in optical rotation, infrared spectra, and certain derivatives (19)[6]; it resembles gibberellin A in biological activity and degradation products. The I.C.I. group repeated the degradation to gibberene, and Mulholland & Ward (97) were able, first, to identify it as 1,7-dimethyl fluorene, and then to establish its structure by synthesis. This led to the proposal by Cross *et al.* (20) of a tentative structure for gibberellic acid and the reaction sequence shown in Figure 1. This structure bears no close relation to any previously described natural product, nor does it resemble any auxin.

The bicyclic unsaturated hydroxy lactone system of gibberellic acid is an unusual, if not unique, configuration proposed to explain the experimental fact that the acid, which has no ultraviolet absorption, readily loses CO_2 in hot acid, producing allogibberic acid whose analysis and absorption spectra indicate the presence of a benzene ring. The exact points of attachment of the lactone to the cyclohexenol ring of gibberellic acid are still not known, and the positioning shown is advanced from theoretical considerations. The

[5] The assignment of the names A and B was reversed in their first papers (177, 181).

[6] This paper by Cross (19) includes a review of much of the Japanese work.

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structures of allogibberic acid, and the gibberic acid produced from it by Wagner-Meerwein rearrangement, seem well established, although the stereochemistry of the molecules is unknown.

The British work was simplified by the fact that their cultures produced only gibberellic acid (13). The U.S.D.A. chemists obtained a mixture of gibberellic acid and a compound similar to gibberellin A which could be separated by partition chromatography (146). In view of this work, Takahashi *et al.* (152) re-examined the University of Tokyo's gibberellin A. Upon

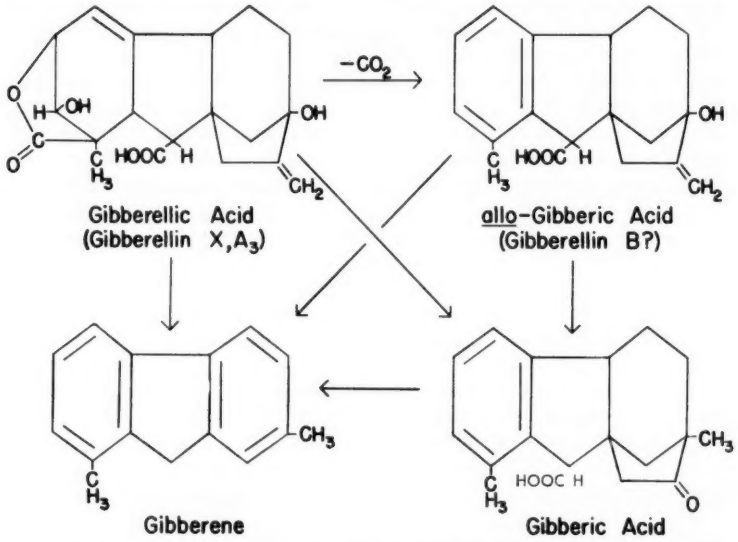

FIG. 1. Structural formulae and relationships of gibberellic acid and its major degradation products as proposed by Cross *et al.* (20).

paper chromatography in ten solvents, partition chromatography in several systems, and by countercurrent distribution, their material appeared homogenous. However, esterification followed by chromatography on alumina led to the isolation of three methyl esters. All three parent acids of these esters have biological activity, and they were named gibberellins A_1, A_2, and A_3. Comparison of A_3 with gibberellic acid showed their identity, and the latter name was accepted by the Tokyo group.[7] It appears that the

[7] The abbreviation GA for gibberellic acid has already been used. Confusion would be avoided if specific gibberellins were identified by subscripts. Thus, GA_3 could be used for gibberellic acid, the others being identified as GA_1, GA_2, GB, GC, etc. until appropriate chemical names are available. Ga has already been used to denote an unspecified mixture of gibberellins (82).

composition of their batches of gibberellin varied from lot to lot, one lot containing less than 5 per cent of gibberellic acid, and another 20 to 30 per cent. Hence the early degradative work must now be reinterpreted bearing in mind that mixtures of gibberellins varying in composition were probably used. It is still not clear why proportions of the components should change in different runs, but this may in part be caused by the variability of the fungus discussed earlier, and to variation in cultural conditions.

All three gibberellins have decomposition points falling within the range 232–237°C., their mixed decomposition points are not significantly depressed,

TABLE I

SUBSTANCES WITH GIBBERELLIN OVERGROWTH (BAKANAE) ACTIVITY

Name	Formula	Free Acid			Methyl Ester		Activity	References
		m.p.*	pK	$[\alpha]_D$	m.p.	$[\alpha]_D$		
		Degrees C.		Degrees	Degrees C.	Degrees		
A_1	$C_{19}H_{24}O_6$	232–5	4.9	+42.3	232–4	+35.1	Strong	152
(U.S.D.A.A)	$C_{19}H_{24}O_6$	255–8	—	+36	—	—	Strong	146
A_3	$C_{19}H_{22}O_5$	235–7	5.4	+11.7	183–5	+28.1	Strong	145, 152
Gibberellic acid	$C_{19}H_{22}O_6$	233–5	3.8	+86	209–210	+75	Strong	19, 202
(X)	$C_{19}H_{22}O_6$	232–5	—	+92	207–8	—	Strong	146
(A_2)	$C_{19}H_{22}O_6$	—	—	—	203–4	+67.5	(Strong)	152
		Less Established Compounds						
B	$C_{19}H_{22}O_5$	197–9	—	−82.3	—	—	Medium	186
allogibberic acid	$C_{19}H_{22}O_6$	200.5–203	—	−80	—	—	None	19
C	$C_{19}H_{24}O_6 \cdot H_2O$	252–4	—	+49.9	222.5	—	Medium	65, 152, 187
"A" (1939–41)	$C_{20}H_{28}O_7$	245–6	—	+36.1	220–1	—	Strong	181, 186
"Dihydro A"	$C_{20}H_{30}O_7$	273–4	—	—	231	—	Strong	186
Derivative of "hexahydro A"	$C_{20}H_{30}O_7$	269–270	—	—	—	—	Weak	186
"A" treated with EtOH-NH_3	$C_{20}H_{31}O_6$	243–5	—	+26	—	—	Medium	186

* Decomposition, except B and allogibberic acid.

and their infrared spectra (152) are closely similar. It is not surprising therefore that their crystalline mixture had so long been considered homogenous. The compounds can be differentiated by their methyl ester melting points, their specific rotation, and their behavior to acid degradation. These properties are summarized in the first part of Table I.

Although the existence of these three distinct gibberellins seems established, only gibberellic acid has been thoroughly characterized. Stodola has concluded that the U.S.D.A. gibberellin A is the same as the Japanese A_1 (146). Their rotations are similar, but the decomposition points differ by 23°C. Decomposition points are notoriously unreliable, but since no detailed

comparison of these compounds has yet been reported, their identity must be accepted with caution.

Gibberellin A_2 has so far only been isolated at Tokyo (152). Stodola reports its corrected formula as $C_{19}H_{26}O_6$ (145). This would make these three gibberellins differ from each other solely by the number of double bonds—an attractive hypothesis—but the fact that the methyl ester of hydrogenated (dihydro) GA_1 has a m.p. of 234–236°C. (65) whereas GA_2 methyl ester has a m.p. of 183–185°C. (152) must be explained. Also methyl tetrahydrogibberellate has a m.p. 270.5–272°C. (20), and cannot be the same as the GA_2 methyl ester. All the methyl esters melt without decomposition so that these figures should be more meaningful than those recorded for the free acids. These melting point differences could lie solely in the stereochemistry, but they emphasize that further evidence is required for the idea that GA_1, GA_2, and gibberellic acid differ solely in their degree of hydrogenation.

The behavior of the three gibberellins to acid degradation is characteristic. Kawarada and colleagues (65) showed that the gibberellin C first isolated from acid treatment of the "A" of earlier work (187) is in fact produced from GA_1. Gibberellin A_2 has not been studied in detail, but its methyl ester in acid reacts differently from the other gibberellins (65, 152). Gibberellic acid (A_3), as mentioned previously, forms allogibberic acid and under more severe conditions, gibberic acid. Cross (19) noting the similar melting point and other characteristics of allogibberic acid to the gibberellin B of the early papers of Yabuta *et al.* (181, 186), compared their infrared spectra and concluded that they were the same. However, Cross also states that allogibberic acid is inactive biologically. Yabuta and his collaborators (186) found gibberellin B active on rice. In serial dilutions to as low as 0.0002 per cent it was consistently about one-tenth as active as their gibberellin A. GB also promoted root growth while Ga did not. Hence their results are not likely caused by contamination. Thus the identity of GB and allogibberic acid should be re-examined, especially with regard to their stereochemistry. This point has importance beyond that of settling the existence of an active compound, since if allogibberic acid is biologically active, then the complex lactone ring of gibberellic acid is not required for physiological action and the synthesis of potentially active analogs would be greatly simplified. The Japanese gibberellin B had ultraviolet absorption (186), so probably contained a benzene ring.

Hydrogenations of the old gibberellin "A" were carried out and some of these led to active compounds (186). Since the nature of the "A" then used is now uncertain, it is impossible to say whether these compounds do match the sequence proposed by Stodola. A "dihydro gibberellin A" had strong activity, whereas "hexahydro gibberellin A" did not. But an acid degradation product of the latter compound was weakly active. Their properties, as reported, are listed in Table I. Unfortunately, no biological testing of recently repeated hydrogenations on better defined material seems to have been reported.

The list of possible gibberellins does not end here. The decomposition point of the original "A" was higher (245–246°C.) than any of the three gibberellins isolated from the more recent fermentations (all within 232–237°C.), so it is possible that another natural compound may exist (65). In addition, a compound from ammoniacal alcohol treatment of "A" was stated to be active (Table I). It should be noted that no accurate comparison of the relative biological activities of these compounds is possible until bioassay methods have been standardized. Besides these fungal products and their derivatives, there is recent evidence, discussed elsewhere, that similarly acting compounds are present in higher plants. No information is yet available on their chemistry.

TABLE II

ACTIVITY OF GIBBERELLIC ACID DERIVATIVES IN PROMOTING STEM ELONGATION OF PEAS*

Esters of		10 p.p.m. to roots†	10 μg. to leaves†
Acid group	Hydroxyl group(s)		
—	Acetyl	++++	++++
—	Diacetyl	+++	+++
—	Butyryl	+++	++++
—	Benzoyl	+++	++++
Methyl	Acetyl	++	+
Ethyl	—	++	—
Ethyl	Acetyl	+++	—
Isopropyl	—	+++	—
Isopropyl	Acetyl	++	—
Butyl	—	++	—
Butyl	Acetyl	+++	—
Octyl	—	++	—
Phenyl	Acetyl	+++	no test

* Adapted from the I. C. I. patent application (202).

† ++++ is equivalent to gibberellic acid.

As with the auxins, some derivatives of the gibberellins retain biological activity. Takahashi and co-workers (152) state that the methyl esters of their three gibberellins were inactive, but do not specify the bioassay used. The I.C.I. (202) tests of derivatives, summarized in Table II, indicate that esterification of the hydroxyl group(s) has virtually no effect on biological activity. Esterification of the acid group prevented action when applied to the leaves, but only reduced the response when applied to the roots. The patent application states that the root treatment response may come about through hydrolysis in the soil.

Chemical assays for gibberellins are in the process of development.

XUM

Kitamura & Sumiki (68) have advanced a polarographic method for gibberellin and several derivatives. Paper chromatographic solvents were used by Kawarada *et al.* (65), West & Phinney (168), and Radley (115). None of these separates the gibberellins from each other, and no entirely satisfactory chemical means of detecting the spots is available. Acid-base indicators, and a fluorescence which develops on treating with sulfuric acid have been used.

The complexity of structure, the possibility of many stereoisomers, and the difficulty of separating and identifying those compounds so far known lead to the conclusion that gibberellin chemistry is unlikely to be rapidly clarified.

The Physiological Action of Gibberellins

Elongation.—The most typical and striking plant response to treatment with gibberellin is stem elongation. Hori's initial paper (51) cited plant measurements 150 per cent of normal, and since then nearly every paper on the bakanae disease or gibberellin has contained measurements of the growth response. In general it is limited to younger tissues which are still growing, varies widely even within a species, and may be influenced by external conditions and the age of the plant. The elongation may result in a change of habit, either forming a vine from a bushy plant (9) or producing a more bushy habit due to outgrowth of laterals (92). Very high gibberellin concentrations are inhibitory, but not always toxic, and the effective range of concentrations is extraordinarily broad. Gibberellin applied to any part of the plant will apparently affect all growing parts throughout the plant. However, gibberellin does not usually change the number of internodes, nor is the growth produced exceptionally abnormal—the general impression is that of more rapid and extensive, but not uncontrolled, growth. Marth *et al.* (92) report that in a number of plants gibberellin becomes less effective with aging, and this supports the general impression provided by other papers that some of the forces that normally check growth are still operative.

The anatomical basis of the increased growth has been the object of several studies. Sawada & Kurosawa (121) could not detect any cell-length differences in infected rice and suggested an increased cell division, but Kurosawa (75) later showed that epidermal and parenchymatous cells of leaf and internode were longer and decreased slightly in their radial and tangential diameters. Shimada (137) compared infected rice and rice treated with the fungal filtrate. In both cases, length of cells was greater than normal, though the ratio varied along the leaf, the leaf sheath being the most affected. The cell-length differences were in a slightly smaller ratio than those of the heights, but he concluded the major part of the bakanae effect was attributable to cell elongation. Stomatal size and distribution were uninfluenced, but guard cell widths were smaller. Imura (55) and Hayashi and co-workers (40) made similar measurements and also concluded cell division was insignificant. Thus in rice there is no doubt elongation predominates.

In other plants Yabuta & Hayashi (178) noted cell elongation, but believe

cell division must have occurred. Brian *et al.* (9) believe cell elongation in peas is sufficient to explain the stem elongation observed, but admit further study is desirable. Y. Kato (64) attributed internode elongation in *Vigna* to cell extension, no division being observed. Gibberellin also strongly promotes pollen tube elongation in *Lilium* (64) but not in cucumber (38).

Yet there can be no doubt that in certain cases, as pointed out by Lang (82) for *Hyoscyamus*, when bolting is induced, cell division must be increased. In a biennial long day plant, Lona (89) found cell length to increase four times in a shoot which lengthened over thirty times. Despite these exceptions, cell elongation in most cases seems to predominate as the result of gibberellin application.

Cell division.—In addition to the above, direct tests for cell division have been made. J. Kato (61) has applied gibberellin in lanolin to decapitated sunflower and tomato seedlings, a procedure in which auxin is very active, and found no callus or swelling to result. Negative responses have been cited in *Bryophyllum* and beans (150), and in tomato and broad beans (9). J. Kato (63) found gibberellin to reduce the callus formation induced by auxin. Wada (167, 150) treated *Tradescantia* stamen hairs with a wide concentration range of gibberellin and concluded that there was no effect on mitosis, even at concentrations toxic to rice. As he noted, his data are too scattered for a decisive conclusion. Experiments showing promotion of elongation of stamen hairs are more convincing. Interestingly, the basal part of each cell in the hair seemed more sensitive to gibberellin and bottle shaped cells were formed. Wada suggested that differences in the cellulose micelle account for the greater growth at the basal end. Y. Kato (64) observed inhibition of mitoses in high (100 mg./l.), but not lower, gibberellin concentrations in two species of *Allium*, and has described the cytological abnormalities produced which were not like those caused by auxin.

Growth of dwarf plants.—A differential response of varieties of plants within one species was noticed in many studies of the bakanae disease. These differences were interpreted as disease resistance variations (122), but gibberellin itself often produces effects dependent on the variety of the species, and Yoshii (199) showed these variations were often similar to the "resistance" of rice varieties.

The most striking instance of varietal response is the effect on certain dwarf plants, first reported by Brian & Hemming (10). They found that a few micrograms of gibberellic acid on a leaflet would increase the growth rate of dwarf peas to that of a tall variety. It is readily possible to obtain differences in height of 500 per cent in a few days after one such application. The growth response was log linear from 0.01 μg. to 2 μg. GA_3 per plant, the length but not the number of all new or growing internodes being increased. Some peas were more sensitive than others, and the response could not be duplicated with auxin. A similar effect was reported for dwarf varieties of *Vicia* and *Phaseolus*. Phinney (114) found that in four dwarf mutants of maize, gibberellin brought growth rates up to that of treated

XUM

normals. Of the other two mutants, one showed slight response, the other none. When treatment was stopped, the mutants slowly returned to a dwarf growth rate. Barton (5) has shown that a nongenetic dwarf also responds to GA_3 treatment. Plants germinated from embryos excised from nonafterripened *Malus arnoldiana* seeds stay in a dwarfed condition unless given a cold treatment. An application of gibberellin, but not auxin, doubled the growth rate of the dwarfs. The growth rate was not compared to that of normal or treated normal seedlings. It should be noted that the effect on long-day rosette plants discussed elsewhere is also in a sense a release of physiological dwarfing.

This response of several mutants, and of environmentally controlled defects in other plants is strong indirect evidence that gibberellin substitutes for some naturally occurring factor in plants. The exact genetic relationships of the dwarf mutants have been discussed by Brian (8). Presumably, since Phinney's maize dwarfs are single gene defects, they may be explained as individual blocks on a biosynthetic pathway to a natural, gibberellin-like, factor. The genetic basis of dwarfing in peas is polygenic, however, and a similarly simple explanation does not seem to be possible. Three nonlinked loci are known to influence the height of peas. The oldest known, *Le*, is found in all normal tall plants, a recessive *le* gene homozygous at this locus causes dwarfing. But the two other loci Cy_1 and Cy_2, modify this response [nomenclature of Lamm (81)]. Both dwarf and tall plants require the presence of either dominant Cy_1 or Cy_2 or both for the *Le* locus to be effective in establishing either the dwarf or the tall habit. Two double recessives are known, because of the presence of two alleles at Cy_2. One, genotype cy_1cy_1 $cy_2^ccy_2^c$, produces a "cryptodwarf" which is readily distinguished from other dwarfs. The other, genotype cy_1cy_1 $cy_2^scy_2^s$, expresses itself in the formation of an unusually tall thin pea plant, named "slender" by de Haan (33). The appearance of this plant is remarkably similar to that of dwarf peas treated with gibberellin. De Haan noted that the plants have more and longer internodes than dwarfs, smaller leaves, fewer leaves per node, reduced fertility and are parthenocarpic. They differ in several respects from tall plants (34), and internodal length increases are attributable to both more and longer cells than in dwarfs [de Haan & Gorter (35)]. Lamm (81) suggested that Cy_1 or Cy_2 can supress internode growth, except in the presence of *Le*. Brian (8) has reported that "slender" plants show no response to gibberellic acid, whereas growth of dwarfs is strongly accelerated. Tall plants show no or little effects; cryptodwarfs were not tested. Brian has tentatively suggested that the action of gibberellic acid on dwarfs is to suppress the action of Cy_1 and Cy_2, and that gibberellic acid and its natural analog are thus "inhibitors of inhibitors." More experiments on plants of definite genotype are highly desirable. The effects of auxin on dwarf plants have been summarized by von Abrams (1), who concluded no simple relation of auxin to dwarfing exists.

Epinasty.—The epinastic responses of tomato leaf petioles, well known to

be very sensitive to auxin, were found quite unaffected by 1 per cent Ga in lanolin (61, 150). A similar response is the increase in the angles between the rice leaf and stem which is one of the symptoms of the bakanae disease. Imura (55) has reported not only that the length of epidermal and parenchymatous cells in the upper surface of rice leaves is significantly longer than those in the lower surface, but that the difference is proportionately greater in infected plants. This results in an increased angle between leaf and stem, which is further enhanced by the weaker mechanical strength of the thinner leaf. Curry & Wassink (21) noted the opposite type of response in *Hyoscyamus* petioles, which were held more erectly after GA_3 treatment, also true of very young infected rice (121).

Leaf expansion.—It is apparent from several studies of diseased rice that the leaves must have increased in total area. Pure gibberellin increases both length and dry weight of rice leaves (40). The effect on other plants is not so clear. In tomato, morning glory, and three Cucurbits, leaf expansion was stated to be inhibited, but at one gibberellin concentration cucumber leaf expansion was promoted (178). Leaf number remained about the same, except in cucumber which was increased. Yabuta *et al.* (184) found the leaves of treated tobacco to be on the whole smaller and paler than controls. But the largest single leaf was found on treated plants, and the number of leaves in two of the four tobacco varieties was more than doubled. Photographs indicate that the experiment was ended when the plants were still young. Another note of the Tokyo group (193) describes tobacco, treated once at an older age, as having reduced total shoot weights at harvesting, though the number of leaves and leaf area are stated to have been larger. The treated plants contained only one-quarter as much nicotine per gram dry weight as did the controls. When tea plants were sprayed with 100 mg./l. gibberellin after the first picking and harvested three weeks later, the total fresh weight and number of leaves were increased by 58 and 28 per cent respectively (195). Two other experiments carried out late in the season after the second picking on another variety of tea showed a decrease in both these quantities, although the fresh weight per bud was higher. In all experiments there are clear differences between the effect on the leaves on upper and lower parts of the stem. The upper leaves were always equal or less in weight than controls, whereas the lower leaves weighed more. Internodes were markedly elongated, and the quality of the tea is stated to be unchanged, although the younger leaves were yellowed and the older leaves wrinkled by the gibberellin. In these tea and tobacco tests, there is no indication that added fertilizer was employed, which as the I.C.I. group has shown (9, 202) is very important.

Yabuta & Hayashi (178) reported that multiplication of the fronds of the aquatic plant *Lemna* was increased by Ga, and Kato (61, 150) found that the increase is the result of more plants, each plant having fewer and smaller fronds. Sunflower and soybean leaf area was also reduced in short-term experiments (61). Lockhart (87) found no effect of gibberellin on the light-

XUM

controlled expansion of leaves on intact peas. Curry & Wassink (21) reported GA_3 reduced leaf number in all their experiments, while Lona (89) describes several leaf effects in his treated plants. Brian *et al.* (9) have noted that their data on wheat and peas indicate a small increase in leaf area.

Hayashi & Murakami (43) studied green and etiolated leaf sections from oats and barley, growing in sucrose. Their results show definite growth promotion, the optimum being at 1 mg./l. Ga, sucrose being required. Several auxins were indifferent or inhibitory at all concentrations, as has often been observed. The age of the leaf section was very important, the youngest sections giving the largest response. Beyond 10 mm. from the leaf base there was no growth promotion with gibberellin, and, in fact, in a narrow zone of 10 to 15 mm., there was growth promotion by auxins. In the presence of auxin the gibberellin effect disappeared in the youngest sections. Murakami (98) has surveyed the effect of many compounds, in addition to gibberellin, on rice leaf-sheath growth. Kuraishi & Hashimoto (72) have found that the expansion of the first leaf of radish seedlings increased up to 25 per cent when floated on the surface of a gibberellin solution. The optimum, at 10 mg./l., was nearly as effective in the dark as in the light, and was not causing cell division. The initial size of the leaves used has to be similar to make comparisons valid. Most interestingly, kinetin promoted leaf expansion similarly, and when supplied with gibberellin, both fresh weight and area increments were nearly the sum of the effect of either compound alone, amounting to nearly a 100 per cent increase.

Radley (115) has proposed a bioassay for gibberellic acid which uses wheat leaf sections. This gives an optimal 40 per cent response over controls to 1 mg./l. GA_3, and a detectable response to 3 μg./l. In view of Kuraishi & Hashimoto's results the specificity of her assay should be examined.

These results on leaf effects of gibberellin are far from conclusive. The effects vary with leaf and plant age, and possibly with season as well. There is no doubt, however, that the capacity to influence leaf growth is one of the gibberellins' major characteristics.

Transport.—Gibberellin apparently does not show the polar movement within the plant which is characteristic of auxin. Studies of this specific point are not yet available, but it is apparent from many reports that treatments by single drop application, with lanolin paste, by spraying, or as a solution to the roots, are all effective on the entire plant. For instance, treatment of one internode (63) or one leaf (72) affected growth of all the younger internodes. The Tokyo workers obtained responses below the site of injection in one experiment (178), but not in another (184). Preliminary results reported by Phinney (113) indicate that transport speed within maize is about the same as auxin at 5 mm./hr.

Roots.—Although the bakanae disease increases adventitious root formation there is general agreement that underground root growth is reduced in length, weight, and root number (51, 57, 75). Fungal extracts act similarly (73, 78, 124, 136, 140, 155, 199). The sole exception was Azuki bean, whose root growth was promoted (136).

When crystalline gibberellins became available much the same results were obtained in solution cultures. The Tokyo group generally found inhibition or no effect (40, 186, 190), nor was branching influenced (150). The I.C.I. workers (9, 202) have obtained consistent root weight decreases in treated peas and wheat, especially in three-week experiments. They point out that nearly all reports are of plants treated via the nutrient solution which could result in local root inhibitions. When gibberellin was applied to peas via the shoot, there were root weight increases. Yabuta & Hayashi's (178) injected plants showed inhibition, however. Thus further work on this point is indicated. Still unsubstantiated are the reports (149, 181, 186) that gibberellin B promotes root growth.

The over-all effect of gibberellin when it stimulates the shoot and inhibits the roots is to cause a marked change in the ratio of shoot weight to root weight. This was noted by Ito & Kimura in diseased plants (57), and can be observed in other data (40, 190) obtained with gibberellin. This may indicate an actual redistribution of material within the plant from root to shoot (9), but whether this is always true is still uncertain.

Critical and specific tests of gibberellin on root growth have only started. The British group (11) found cress roots not influenced by GA_8 up to 100 mg./l. It had little effect on IAA root inhibition, although there were statistical indications of slight interaction. J. Kato (63) could find no effect of gibberellin on cucumber root growth, nor did it modify the inhibition caused by IAA, naphthalene acetic acid, maleic hydrazide, or coumarin. Y. Kato (64) found little gibberellin effect on roots of onions, but rhizoid cells of ferns were strongly promoted.

As noted above, diseased plants often form adventitious roots and Mostafa (94), showing that fungal extracts can cause root formation on disease-resistant tomatoes, suggested this rooting ability was the reason for their resistance. But tests of gibberellic acid show it reduces the natural rooting of peas (11) as well as the root promotion caused by auxin (8, 63).

Chlorosis.—The typical yellowing symptom of the bakanae disease is also brought about by gibberellin. Fewer chloroplasts are present in diseased tissue (75) and the chlorophyll decreases (57). Gibberellin treatments also decrease the per cent chlorophyll (190). It is possible to calculate from the data that the rate of chlorophyll formation must have been less in treated plants. Oddly enough, there is one report (69) of chlorosis, brought on by another fungus, being reduced by *F. moniliforme.*

The I.C.I. group (9) showed that increased nutrients markedly reduced gibberellin chlorosis. A field experiment (202) indicated that added fertilizer must accompany gibberellin application if chlorosis is to be avoided. It would be interesting to know which nutrients are most important.

Metabolism.—When gibberellins promote elongation they do not always cause a parallel increase in dry weight. Ito & Kimura (57) found bakanae plants had increased in fresh weight, but dry weights were less, whereas some of de Haan's (36) and Edwards' (26) diseased plants definitely showed dry weight increases. Shimada (136) showed that elongation paralleled fresh

XUM

weight increases in several plants, the exception being Azuki bean. Others have found increases with tea (195) and isolated radish and pea leaves (72), increase (190) or no consistent effect (194) in rice, and decreases in tobacco (193). Hayashi *et al.* (40) showed that dry weight differences required at least two days to appear, and followed the changes with time in rice (44). The most detailed studies of dry weights are those of the I.C.I. group (9) who show that the total dry weight of wheat and peas is increased by gibberellic acid treatment, provided that sufficient nutrients are supplied and the experiments cover a long enough period. They also make the important point that redistribution of material from the root to the shoot must be taken into account. Although other constituents vary in minor ways, the increased dry weight was traced to more carbon fixation, and the largest carbon changes involved carbohydrates. Recent reports emphasize the complex nature of the effect on dry weight (13a, 93a).

Carbohydrate changes were encountered in microscopic examinations of diseased plants, which revealed decreases in starch (75, 121). This was later confirmed by direct measurement (40, 190). These Tokyo papers also indicate a decrease in total sugars, dextrin, reducing sugar, and sucrose in treated rice plants. On the other hand, the British group (9) reports marked increases in sucrose, fructose, and glucose in wheat; in peas only glucose was affected. No starch was found, and it was suggested protein or cellulose must increase. Hayashi and colleagues (40) noted an increase in cellulose and hemicellulose and a drop in protein (44). Nitrogen levels (9, 40, 44, 190, 195), showed inconsistent fluctuations, but these make it unlikely any major increase in protein occurs.

It should be noted that this effect of gibberellin on carbon fixation is not directly related to photosynthesis, since the bakanae effect occurs in the dark (87, 178, 192). Also, Hayashi (39) found the amylase activity of germinating barley and wheat (but not rice) seeds was increased on a weight basis proportionally to the applied gibberellin concentration. The enzyme decreased in treated seeds when they began to grow. The enzyme alone was not activated by gibberellin. Recently, Hayashi *et al.* (44) have examined changes of enzymatic activity with time during the development of the seventh leaf of rice. Phosphatase, alkaline pyrophosphatase, acetylesterase, maltase, β-glucosidase, α-galactosidase, amylase, urease, dipeptidase, ascorbic acid oxidase, and catalase decreased on a fresh weight basis more rapidly than in nontreated controls. But peroxidase and invertase increased more rapidly than controls and gibberellin was shown to have no effect on their action. Qualitatively, however, none of these enzymes had strikingly different patterns of change from those that occur in normal leaves, the changes were simply more rapid and more marked. An increased peroxidase and "oxidase" activity in treated tea had also been shown earlier (195). No change was found in the osmotic pressure of rice leaf sheath cells followed over a 48 hr. period (40).

The ascorbic acid content of etiolated soya beans was slightly increased

by gibberellin treatment (192), but asparagine and reduced ascorbic acid were unchanged and the latter was also unaffected in tea (195). The levels of caffeine, tannins, and raw fibers in tea showed no consistent differences. Nicotine was reduced nearly 75 per cent in treated tobacco (193). Ash (9, 40, 190), potassium and phosphorus (9) levels have been followed but no strong trends can be discerned.

The effect of gibberellin on plant respiration has not yet been reported in detail. Baldacci (2) attempted to measure the CO_2 output of diseased plants, but his method was unreliable. Hayashi (38) found no effect of gibberellin on yeast fermentation, but did find a slightly greater dry weight loss in treated germinating seeds (39). Kato (62) lists a 15 per cent increase in oxygen consumption in growing pea stem sections; the promotion was not found in nongrowing tissue. He also (63) found that cyanide, arsenite, and *p*-chloromercuribenzoate inhibited gibberellin-induced pea stem elongation in low concentrations whereas copper enzyme poisons did not. He concluded gibberellin did not change the terminal oxidase of the tissue. Wittwer *et al.* (171) reported a marked oxygen uptake in treated bean stems, accompanied by greater activity of pectin methyl esterase, phosphatase, and β-amylase. All these data are reminiscent of the findings with auxin.

A most interesting metabolic effect was reported by Hayashi & Murakami (41) who found that L-tryptophan, the only one of a wide variety of compounds, increased the growth response to gibberellin. They also found that pea sections, starved in distilled water for 18 hr., lost their response to gibberellin while retaining their sensitivity to auxin. Although sugars did decrease in this time added sucrose did not restore the gibberellin activity, even though sucrose had improved the response of nonstarved sections. However, tryptophan was able to restore part of the sensitivity of the starved sections to gibberellins. The effect of tryptophan was also shown in nonstarved tissue, 10 mg./l. tryptophan and 6 mg./l. Ga in 2 per cent sucrose showing an effect much larger than the sum of the response of either tryptophan or Ga alone. Their sample of tryptophan alone was relatively free of auxin contamination, since 10 mg./l. alone showed no significant growth. Other experiments indicated an auxin-gibberellin synergism in starved tissue. They suggested that gibberellin promotes the conversion of tryptophan to IAA, creating the same augmented synergistic response. If true, this would explain the action of gibberellin in terms of auxin synthesis, an important point which requires further investigation. Baldacci (4) has also suggested that an influence on auxin metabolism is involved. With starved wheat coleoptiles, results were less striking, although at 4 mg./l. Ga and 10 mg./l. tryptophan there was a more than additive response (42). In oats only the youngest tissue responded to gibberellin. Tochinai & Ishizuka (161) showed that the effects of one application of fungal filtrate had disappeared by harvest time and others have stated that gibberellin applications must be continued to maintain their effect (114). The quantitative data of Brian & Hemming (10) demonstrate that considering the minute quanti-

XUM

ties of gibberellic acid involved the compound is remarkably stable within the plant.

Flowering.—In the first definitive description of the bakanae disease Hori (51) noted that those infected plants which reached adult size fruited earlier. Kurosawa (75) similarly noted earlier flowering; but no study of flowering as such appears in the Japanese reports. The Tokyo group (184) tested gibberellin on tobacco inflorescences and found them to grow faster and longer, but no comparison of flower number or fruiting was made. Their photographs indicate that in another experiment controls still had a rosette form while treated tobacco plants were elongating markedly, but no effect on flowering is stated.

Lang (82) treated a biennial variety of *Hyoscyamus* with Ga and found that plants in both long and short days bolted the first season. The effect was more marked in long days and repeated applications of Ga were required, but the treatment produced plants that normally would only have developed following a cold treatment. Auxin was without effect. Lang initially proposed that gibberellin was substituting for the cold treatment, but later (83) he found an annual variety not only flowered and fruited on long days but also, more slowly, formed flower buds on short days (14a, 84), so that apparently the long-day requirement was also replaced. The extension of this phenomenon to biennial plants generally is being actively investigated. Bünsow & Harder (14) found that two species of *Bryophyllum*, which do not require a cold treatment, but long days followed by short days, for flowering, could be induced to flower with gibberellin after years spent in short days. Lang has reported (84) three other long-day plants, annual *Hyoscyamus*, *Silene*, and *Samolus*, could be brought to flowering with gibberellin.

Lona (89) induced five more long-day plants to elongate as if forming flower stalks with GA_3, but only one, *Crepis*, formed flowers in short days, while another, *Lactuca scariola*, did not flower on this stalk even in long days. Two short-day plants elongated, but did not flower in long days, while in short days the flowering of one of these was reduced. Recent papers, received too late for comment, deal further with flowering (14a, 14b, 84a).

The relationship of these results to light effects on flowering is being sought. Curry & Wassink (21) showed that gibberellic acid induced shoot growth and flower buds of *Hyoscyamus* in long days at all wavelengths tested; flowers failed to form only in green light. It thus replaced the far-red light required for flowering. Their results and those of Lona & Bocchi (90) with *Perilla* indicate that the red light inhibition of growth is removed by GA_3, and that to some extent GA_3 augments the far-red response.

Wittwer & Bukovac (170) have investigated varietal differences within a species to the flowering response. They found that in beans and tomatoes flowering was only hastened in determinate varieties, although elongation was promoted in all plants. No reduction in internode number resulted from earlier flowering since growth of the plant was faster. Lettuce bolted and flowered as if normally induced, whereas cabbage formed a seed stalk but

no flowers. Marth *et al.* (92) noted the same behavior in cabbage as well as in beets. They found flowering hastened in dahlia, petunia, and salvia, but retarded in geranium and peppers. In general, the data suggest that gibberellin acts primarily on elongation, incidentally releasing the flowering response in some cases, rather than directly on flowering itself. But it is likely that gibberellin will strongly influence future theoretical work on flowering, and very likely will lead to practical applications.

Fruiting.—The bakanae disease is frequently cited as reducing fruit yields, in fact the majority of the colloquial names for the disease refer to this trait. Hori (53) and Ito & Kimura (57) have cited data showing fewer grains per ear. There is some evidence that the gibberellins themselves can cause reduced grain production; Hayashi *et al.* (40) found 2 mg./l. Ga reduced rice grain production 32 per cent, although the yield of straw increased by 14 per cent. Lang (83) noted that high levels of gibberellin had a toxic effect on the bolting stem. Wittwer & Bukovac (170) found gibberellin to be some 500 times more effective than IAA in inducing parthenocarpy of tomatoes, but do not state its effect on fruit yield. Experimental work on fruit yields will doubtless require that toxic levels be carefully established and ample nutrients supplied before valid conclusions can be drawn.

Seed germination.—It has been frequently observed that seed germination time is reduced by gibberellin treatment. Hayashi's data (39) show that barley and rice germinated more rapidly as gibberellin concentration was increased, but since final germination percentage was nearly complete in all cases, no effect on total germination can be discerned. Dry weight loss was only slightly more in treated seeds. Y. Kato (64) indicates fern spore germination was hastened by gibberellin treatment. The relationship of the germination promotion to the well known red, far-red light effect in lettuce seeds was investigated by Kahn *et al.* (59). They found, as also did Lona (88), that gibberellin could substitute for red light in promoting lettuce germination but this chemical promotion was not reversed by far-red. It may tentatively be supposed then (cf. 39) that gibberellin does not specifically act on the light receptor, but rather as a general germination stimulator. Lona (88) has pointed out that this conclusion is also supported by the fact that red light and gibberellin have opposite effects on shoot growth, and so would not be expected to act in the same way on germination.

Light effects.—A connection between the action of gibberellin and the action of light seems apparent from even a brief comparison. The etiolated appearance of treated plants suggests a reversal of the light inhibition of growth, the chlorosis an inhibition of the light-promoted synthesis of chlorophyll, and the increased carbon fixation a relationship to photosynthesis. Also, both light and gibberellin promote leaf expansion, gibberellin can substitute for the long-day requirement for flowering in some plants, and both gibberellin and light strongly influence the growth of dwarf peas. It was also noted above that the effects of gibberellin and of light are not always in the same direction, so that a simple alliance is not likely.

Further examination of this attractive relationship points up difficulties.

XUM

Some effect of gibberellin is manifested even in the dark (87, 178, 192). Furthermore, those gibberellins so far characterized have no absorption in the visible or the ultraviolet (19, 152), although gibberellin in alcoholic solution could be destroyed by strong ultraviolet radiation (189).

Lockhart (87) has examined gibberellin effects on the red light inhibition of pea stem growth. Using plants treated at a time of great sensitivity to red light, he was able completely to reverse the red light inhibition by application of gibberellin, but not with auxin. Strictly speaking, the growth of treated dark plants matched that of treated light plants, but these were both greater than untreated dark plants. Lockhart believes this indicates that an endogenous gibberellin-like factor is limiting growth in dark plants. Because gibberellin did not interfere with two other light-influenced processes—internode number and leaf expansion—its effect on elongation is viewed as relatively specific and not directly connected with the light response inasmuch as the action spectrum for leaf expansion is similar to that for stem elongation. The growth rate of dwarfs treated with Ga was also shown to be similar in light and dark. Vince (164), however, has recently compared the effects of light on the growth of dwarf and normal peas, showing they differ from each other and from tomato in their response to light energy and wavelength. Any definitive explanation of the light effect will also have to explain these differences.

Natural occurrence.—There is little doubt that the gibberellins must correspond in their action to naturally-occurring compounds in higher plants. Not only is their activity a promotion of natural processes, but if the one gene-one biochemical reaction theory is valid for the dwarf maize mutants, then gibberellin must be substituting for the missing product of this reaction. The gibberellins also influence the growth of an extraordinarily large group of plants. Table III, which summarizes those plants on which gibberellin has so far been tested, includes more than 80 species from widely separated families.

There is also direct evidence for the existence of gibberellin-like materials. In 1951, J. W. Mitchell *et al.* (96) described a material obtained from ether extracts of recently fertilized bean seeds, which can now be interpreted as similar to gibberellin. When applied to bean seedlings, it increased growth of all internodes even if applied only to the first, increased speed of leaf expansion and final size of leaves, caused earlier flowering, was more effective on young plants and on growing parts of plants, and reversed the light inhibition of growth. These effects were not duplicated by auxin. Significantly, the bean seeds only produced this material for a few days, the sharp maximum being at seven days after pollination. More recently, West & Phinney (168) have obtained ether extractable material active in promoting growth of dwarf maize mutants from many plants. Wild cucumber seed was the most active, one *Echinocystis* seed yielding material equivalent in activity to 5 μg. of GA_3. Their methods did not reveal active factors in other plants. Paper chromatography showed that several substances are involved, but only ma-

terial from beans gave R_f values similar in some solvents to that obtained for the known gibberellins (which all have the same R_f value in all solvents so far reported). Similarly, Radley (115) has obtained material from pea shoots which is active in a wheat leaf-sheath assay, and which runs at the same R_f as gibberellic acid. A leaf growth-promoting factor has long been known to occur in peas (7).

The fact that the material of Mitchell *et al.* was best obtained from seeds soon after fertilization makes it possible that gibberellin is analogous to hormonal material whose presence in the plant has long been suspected. Briggs and collaborators (12) pointed out that maxima occur in plant growth at flowering, and Murneek (99) showed that removal of developing tomato fruits shortly after fertilization led to growth stimulation of the entire plant. This did not occur if fruits were left on or if flowers were removed prior to fertilization. The greatest differences in his analyses of this effect were in the total carbohydrate figures. Dearborn (23) obtained similar results with cucumbers. Wittwer & Murneek (172) showed that substitution of auxin for removed fruit was only partially effective. The reinterpretation of the relationship of these and other suggestions of real or postulated growth substances such as vernalin, florigen, syngamin, coconut milk factors, and caulocaline in terms of possible gibberellin components is likely to lead to further insights into plant growth regulation.

Relationship to auxin.—Although the gibberellins promote cell elongation, it is apparent that they differ from previously known auxins in several respects. They do not in short term tests inhibit root growth, they inhibit rather than promote root initiation, they do not cause typical epinasty nor callus formation, their action on leaf growth or dwarfism is not paralleled by auxins, and they are more potent than auxin in inducing parthenocarpy. Several other properties of auxin have been compared with gibberellin. Brian *et al.* (11) could find no effect of gibberellic acid on water uptake by potato tuber disks, nor did it cause any delay of leaf abscission. Also it broke potato dormancy. All these actions are contrary to those caused by typical auxins. Similarly lateral buds were not inhibited but strongly promoted by gibberellic acid, a result previously observed by Kato (61, 150). Perhaps the most striking of all the differences is the evident indifference to light of the gibberellin effect. So there is good reason that ever since the first comparison (176) investigators have agreed that gibberellins are not auxins in the usual sense of the term.

Yet those gibberellins so far tested do meet the requirements of activity in some of the auxin bioassays and strictly speaking are thus auxins, albeit atypical ones. In the most classical auxin bioassay, the *Avena* curvature test, the gibberellins have so far given negative results (61, 150), as also in the pea curvature test. Nonpolar transport may explain the failure of the first of these. But in straight growth tests of isolated sections they are fairly active. Pea epicotyl sections at optimal gibberellin concentrations extended about 50 per cent of the maximal obtained with IAA and this paralleled fresh

XUM

TABLE III

HIGHER PLANTS REPORTED TREATED WITH THE GIBBERELLINS

Species	Treatment*	Action† and Reference
Acer palmatum	G	B, 92
Acer saccharum	G	B, 92
Ageratum houstonianum	G	b, L+, 92
Allium cepa	G	C, 64; N, 92
Allium fistulosum	G	B, C, 64
Apium graveolens	G	B, p, 92; M, 13a
Arachis hypogaea	G	B, 92
Aster sp.	G	B, 92
Avena sativa	G	b, 42, 108; p. 43; N, 61
Beta vulgaris	G	B, (F), 92
Brassica oleracea	G	B, (F), 92v, 170
Brassica napus	G	b, 178
Brassica rapa	G	b, 92; (F), 84a
Bryophyllum sp.	G	F+, 14; N, 150
Buxus sempervirens	G	B, L−, 92
Callistephus sp.	G	B, F+, 70
Capsicum frutescens	G	B, F−, 92
Circaea lutetiana	G	B, p, 89
Citrus sp.	G	B, L+, 92
Coleus sp.	G	p, 11
Crepis leontodontoides	G	B, F+, 89
Cucumis sativus	G	B, 9, 63, 178; M, 63; p, 178; SN, 38
Cyathea sp.	G	S, 64
Dahlia sp.	G	B, F+, 92
Dryopteris erythrosa	G	S, 64
Euphorbia pulcherrima	G	B, 92
Fagopyrum esculentum	G	b, 178
Geranium sp.	G	B, F−, 92
Gladiolus sp.	G	N, 92
Glycine max	E	B, 178; b, 136, 138
	G	B, 61, 92v, 150; b, 192; p, 61; M, 192
Helianthus annuus	G	B, 61, 92, 150; p, 61, 150
Hordeum vulgare	E	b, 136, 138
	G	B, p, 43; b, 92; S, 39v
Hydrangea sp.	G	B, 92
Hyoscyamus niger	G	B, F+, 21, 82, 83, 84, 84a
Ipomoea nil	G	B, p, 178
Juniperus chinensis	G	B, 92
Lagenaria leucantha	G	b, 1, 178
Lactuca sativa	G	(F), 170v, 171; S, 59
Lactuca scariola	G	B, (F), 89; S, 88
Lemna paucicostata	G	B, 150, 178; p, 61
Lepidium sp.	G	N, 11, 148
Lilium longiflorum	G	S, 64
Linum usitatissimum	G	b, 9, 92
Liriodendron tulipifera	G	B, 92
Luffa cylindrica	G	B, p, 178

* G = gibberellin
E = fungal extract

† B = bakanae overgrowth >50%
b = bakanae overgrowth <50%
C = cytological observations
F− = flowering delayed
(F) = partial promotion of flowering response
F+ = flowering promoted
L± = laterals promoted or inhibited
M = metabolic effects
N = no effect
p = leaf observations
S = spores, pollen, or seeds treated
v = more than one variety tested
W = wounding improved response

TABLE III—(*continued*)

Species	Treatment*	Action† and Reference
Lycopersicum esculentum	G	B, 9, 61, 92, 150; b, 178; p, 61, 178; F+, 170v; N, 11; M, 13a
Malus arnoldiana	G	B, 5
Malus sylvestris	G	b, 92
Nicotiana rustica	G	B, p, (F), 184
Nicotiana tabacum	G	B, 92, 184v, 193; (F), 184v; M, 193
Oryza sativa	E	B, 2v, 45, 48, 56, 73v, 75v, 78, 79, 80, 124, 125, 127, 137, 138, 140, 148, 154, 155, 161, 175, 177, 196, 199; F+, 75; p, 73, 75, 78, 124, 125, 137, 154, 155; C, 75, 137
	G	B, 40, 44, 152, 174, 176, 178, 179, 181, 187, 189, 190, 194; M, 39, 40, 44, 190; S, 39; C, 40; p, 40, 98
Paulownia tomentosa	G	B, L, 182
Penstemon antirrhinoides	G	B, L+, 92
Perilla ocymoides	G	B, 89v, 90; F−, p, 89v
Petunia sp.	G	B, F+, 92
Phaseolus angularis	E	B, 136, 138
	G	B, 178
Phaseolus vulgaris	E	B, F+, p, 96
	G	B, 9, 10v, 92v, 95; L+, 61, 150; F+, 170v
Phaseolus radiatus	G	b, 178
Picea glauca	G	b, W, 92
Pinus strobus	G	N, 92
Pinus taeda	G	b, 92
Pinus virginiana	G	b, 92
Pisum sativum	G	B, 9, 10v, 11, 13a, 41, 62, 63, 72, 87; M, 9, 13a, 41, 63; L+, 11, 150; p, 72
Populus sp.	G	b, W, 92
Quercus phellos	G	B, W, 92
Raphanus sativus	G	p, 72
Rhododendron catawbiense	G	B, 92
Rosa sp.	G	B, 92
Saccharum sp.	G	B, 92v
Salvia splendens	G	B, F+, 92
Samolus parviflorus	G	F+, 84, 84a
Secale cereale	G	(F), 84a, 89, 91
Silene armeria	G	F+, 84, 84a
Solanum melongena	G	B, 92
Solanum tuberosum	G	B, 92; S, 11
Thea sinensis	G	B, p, M, 195
Tinantia fugax	G	B, p, 89
Tradescantia reflexa	G	C, 150, 167
Trifolium sp.	G	b, 9
Triticum vulgare	E	B, 199; b, 136, 138; p. 115
	G	B, p, 9; b, 11, 13, 42; M, 9, 42; S, 39
Vaccinium corymbosum	G	b, 92
Vicia sp.	G	B, 10; CN, 11
Vigna sesquipedalis	G	B, C, 64
Zea mays	E	b, 73, 136, 138; B, 157
	G	B, 113v, 114v; B, 92

weight increases (41, 62). In sections starved 20 hr. in water, there was synergism between gibberellin and IAA (41). In wheat coleoptiles (42) no response was obtained, although a small synergism was noted with trypto-

XUM

phan in starved sections. In oats only the youngest coleoptile tissue showed a gibberellin response. At low concentrations (0.01 mg./1.) GA_3 has a stronger effect on wheat coleoptiles than does IAA, but this effect is small and does not increase at higher concentrations where IAA is much more effective (11). In pea stem sections, GA_3 was reported more active thar IAA, but over-all growth was mediocre (11). The differing results of the Japanese workers were obtained with another gibberellin, and in some experiments, sucrose was added. The proposed structure for gibberellic acid would not be expected to have auxin activity on the basis of the many studies of auxin structural requirements. In fact, unsaturated lactones are usually inhibitors of growth.

Interaction, or synergism, between IAA and gibberellin is still not clearly established. Kato (63) obtained better growth with IAA than with gibberellin in pea sections, but found that the smaller effect of gibberellin was additive to the IAA (or naphthalene acetic acid) response at all concentrations. Pea cuttings in sand showed marked intereaction between the two hormones, the height of the plants being markedly greater in 10^{-5} *M* GA_3 and 10^{-5} *M* IAA than in either alone or in any other combination tested (8). Nitsch & Nitsch (108), using oat coleoptiles and first internodes, found that IAA and gibberellic acid mixtures generally showed less than additive growth responses, but in two combinations did show synergistic increases. It may be important that Hayashi & Murakami (41) found their synergism in starved tissues. Thus, the testing of gibberellin in auxin bioassays has yet to explain all the facts observed.

Some attempts have been made to study synergism in the intact plant. The Tokyo group (194) studied rice seedlings to which gibberellin-auxin mixtures had been applied in different ways, but their results were not uniform enough to permit conclusions. Kato (63) found that in cucumber seedlings gibberellin reduced the IAA or naphthalene acetic acid inhibition of growth at all concentrations. Of interest here is the report by Marth *et al.* (92) that wounding of several plants increased the gibberellin response, suggesting a synergism with wound hormones. Studies of auxin-gibberellin interactions, and of the effects of gibberellin on auxin synthesis, should eventually explain why gibberellins possess auxin assay activity.

Bioassay and terminology.—Despite the broad variety of effects caused by the auxins, their definition rests largely on a single property, that of cell elongation [cf. Tukey *et al.* (162)]. This was selected partly because it was common to many auxin effects, but also very importantly, because it could be rigorously measured. Until the specific site of auxin reaction is identified, any definition will remain in part ambiguous, and the same is true for gibberellin. Since gibberellins cause distinct effects in plants, and some members of the group are probably present in higher plants, it seems proper to suggest an operational definition for them. The one property common to most of the gibberellin effects is their removal of certain limitations on cell elongation. Whether this is by suppression of an inhibitor or an inherent stimulation is as

yet uncertain, but in shoot elongation, leaf expansion, growth of dwarfs, parthenocarpy, bolting of long-day plants, or removal of light inhibition, gibberellins seem to be removing a normal limitation. The reported effects on root growth, cell division, chlorosis, and metabolism would seem secondary.

The question is then in which response is gibberellin most specific? Of those listed, at the moment the growth of genetic dwarfs seems most promising, since we may assume from biochemical genetics that these plants contain the smallest amounts of endogenous gibberellin. And indeed the response of these dwarfs is extremely sensitive, Phinney having reported that 0.001 μg.[8] of gibberellin per maize plant produces a measurable effect. Dwarf peas would probably be the most readily available material for bioassays. Radley's (115) leaf section bioassay is also extremely sensitive and has the virtue of simplicity, but may not be specific. It may be possible to utilize some plant materials by adding other hormonal factors such as auxin or kinetin to reduce variability, or, if synergistic, thus increase the response. When the light response is better understood, a test utilizing the removal of light inhibition may also be devised.

For the present it is suggested that gibberellins be defined as that class of compounds which cause internodal elongation when applied to certain intact genetically dwarfed plants. A promotion of elongation of monocotyledenous leaves might provide a useful supporting definition. It may be objected that the class name gibberellin, despite its twenty year priority, is not sufficiently descriptive to justify its retention. However, by chance, *Gibberella* derives its name from the Latin word for hunchback, and hence the use of the response of dwarf plants as an operational definition for gibberellin seems singularly appropriate.

Agricultural utility.—No specific use of gibberellin can now be recommended for agricultural purposes, but certain applications can be anticipated. Although the first attempts to vernalize grain with it failed (84a, 89, 91), other conditions may well succeed, and it seems likely that some biennial crop will succumb to gibberellin treatment, especially for breeding purposes. The few Japanese reports on crop yields are disappointing and application to fruiting crops will have to be carried out cautiously to avoid outweighing favourable effects with deleterious ones. Leafy crops, such as tobacco and tea, have been shown to be influenced by gibberellins, but whether this influence can be made wholly favorable remains to be seen. The most immediate promising area of utility would seem to be increasing the yield of carbohydrates and cellulosic materials. The I. C. I. patent application (202) cites a case where 4 oz. of GA_3 per acre increased the yield of hay from 10.0 to 18.7 hundredweights per acre; with the addition of six hundredweights of fertilizer per acre the yields were 18.3 and 23.3 hundredweights of hay without and with gibberellic acid respectively. Since the fungus producing the

[8] Sic, the figure cited in (113) is a typographical error.

XUM

substance grows on almost any substrate, the price should not be prohibitive when mass production is attained. If gibberellin can be produced at the cost of penicillin (especially since there will be no need to prepare it in a medicinally pure form) then it can be calculated that the value of hay obtained above would be of the same order of magnitude as the cost of the gibberellin. For more valuable crops there would certainly be more justification for its use. In any case, the requirements for additional nutrients, the age of the plants at application, and the number of applications required will have to be taken into consideration. Horticultural applications, especially with biennials, can more readily be envisaged and will doubtless appear rapidly. The spectacular increase in the growth rate of some trees (92, 182) will certainly be examined further.

Action in other than higher plants.—Reminiscent of the auxins, no convincing action of the gibberellins has yet been shown on any organisms except higher plants. The British workers (9, 13) tested eleven bacteria and thirteen fungi with negative results; they also showed gibberellic acid is microbiologically inactivated in the soil. However, *G. fujikuroi* has been suggested to be antagonistic to several other fungi (26, 69, 142). Stoll (148) used an *Ustilago* spore germination assay for gibberellin with inconclusive results. There was no effect of gibberellin on yeast (38). Ono (109) showed *G. fujikuroi* filtrates promoted sclerotium formation in two other fungi.

Fukuoka (30) concluded there was no effect of gibberellin on chicken heart tissue cultures, as did Kawarada *et al.* (65) in examining its influence on growth of chicken embryos within the shell. Data of these experiments is not uniform, however. No toxicity studies of gibberellin are available, but it should be noted that Kambayashi & Otake (60) inoculated the fungus on guinea pigs without marked effect. Nevertheless, some of the animals fed fungal cultures by Steyn (144) were paralyzed and died. The first description of *F. moniliforme* is due to the fact that plants infected with it were poisoning farm animals (135).

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XUM

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[10] See also reference 179.

STOCK AND SCION RELATIONS[1]

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The composite plant formed by grafting a scion on a rootstock provides a unit of considerable scientific interest and commercial importance. The combination of two plants of different genetic constitution, thus having different inherent characters, affords new opportunities of studying these characters, and of making wider use of them.

One of the first general reviews of rootstock/scion relations was that of Hatton, in 1930 (1). This included much of his own pioneer work, which led to a great expansion of interest in clonal rootstocks (2, 3). Tukey & Brase (4) included a useful review in a paper on the influence of scions and interstocks. Hilkenbäumer (5) reviewed apple, pear, and plum rootstock results in Germany in 1953, and Hoblyn (6) gave a brief practical summary of results in England up to 1950. Tubbs (7) in a general review of work at East Malling, illustrated the extensive range of rootstock effects with a list of 29 scion characters found to be influenced by the rootstock. Reviews on special aspects of the subject include those of Argles (8) on stock/scion incompatibility, and Graves (9) and Katyal (10) on double working; Roberts (11), writing on "Theoretical aspects of graftage," provided a comprehensive catalogue of 479 references with concise comments on the papers cited. The present paper is not intended to cover as wide a range, but will deal mainly with the part played by different components of the composite tree, incompatibility, viruses and certain other aspects, concluding with a discussion of some possible mechanisms of rootstock effect.

At the outset, it must be said that the discovery of latent viruses in some clonal rootstocks and scion varieties has introduced a new factor of uncertainty into some past rootstock trials. Varying proportions of certain clones, particularly M.IX and M.I, were found to be infected with rubbery wood virus, which has been shown to have a dwarfing effect [Posnette & Cropley (12, 13); Luckwill (14)]. Further work by Posnette & Cropley with apple mosaic (15), cherry (16), and pear (17) viruses has shown marked dwarfing effects, both with and without leaf symptoms.

It is reassuring, however, that tests of each tree in the classical Malling apple rootstock trials have shown that the original clones were evidently not infected with rubbery wood or mosaic and that the original conclusions on rootstock vigour remain valid (18).

In discussing other papers it can only be assumed that the rootstock results are not clouded by virus infection; but the importance of using only virus-tested material in future is evident.

[1] The survey of literature pertaining to this review was completed in November, 1956.

XUM

The Relative Importance of Root and Stem

Recent work has gone some way toward resolving the old controversy on the parts played by root and stem in rootstock effect; new interest has also arisen in the control of vigour and fruitfulness by the use of interstocks[2] (intermediate stempieces) and by bark rings. For a correct assessment of the likely effects of these practices the functions of the different parts of the tree must be kept clearly in mind.

In general, the rootstock acts as the absorber of all the mineral elements and water, and the scion, with its leaves, is responsible for the assimilation of carbon dioxide and the building up of carbohydrates and most of the auxins. The rootstock stem, or any interstock, can presumably only affect the tree by limiting—or possibly in some cases aiding—transport of materials, or by modifying these materials in the course of transit. These points will be considered later.

The influence of rootstock roots compared with rootstock stems.—Beakbane & Rogers (19) found that the typical influence of some clonal apple rootstocks was obtained in trees in which no rootstock stem was present. The trees were bench-grafted direct on clonal piece roots. When the growth of this series was compared with that of stem-grafted trees, it was seen that the presence of a piece of rootstock stem intensified the rootstock effect, but its influence was small compared with that of different rootstocks.

Influence of scion roots.—Another good example of the predominance of root over stem influence is provided by the striking changes in growth which may occur when a fruit tree forms scion roots. If the apple Bramley's Seedling on the dwarfing rootstock M.IX forms scion roots, for instance, it grows away very vigorously; and similarly, scion rooted pears, originally on quince rootstocks, grow more vigorously and uprightly, with delayed fruiting (20, 21). In such cases, of course, the stem has been present all the time, but the change in vigour only occurs when scion *roots* form. Scion rooting has even been made use of by some growers who have planted apple trees on M.IX, taken the early crops induced thereby, and later "earthed up" the unions to induce scion rooting and increased vigour; and the same practice has been suggested for pears in France (22).

Inarching.—Inarching a more vigorous rootstock into the stem of an established tree, is another practice which can be used to increase vigour by providing a supplementary and more vigorous root system plus a short piece of rootstock stem (23, 24).

The effects of interstocks will be discussed later.

The relative influence of rootstock and scion on growth.—Vyvyan (25) has shown that although the growth of the composite tree is a compromise between the growth rates of rootstock and scion, the influence of the rootstock on the relative growth rate is greater than that of the scion. This result

[2] In this review the term interstock will be used rather than the longer term "intermediate stempiece."

was obtained from single-worked trees in which three apple clones normally used as rootstocks were combined as scion and rootstock in all nine possible ways. Sample trees were harvested at intervals over a period of three years. The relative weights of the component parts of each tree on the occasion of each harvest, and their relative growth rates between harvests, were obtained by calculating the ratios between their geometric mean weights. The ratio of the product of these for trees with unlike unions of the component varieties, e.g., IX/XII×XII/IX, to that for trees with like unions was slightly, but not significantly, less than unity, indicating that the growth rate of the composite tree is mainly the resultant of the growth rates of the component varieties. Composite trees, e.g., IX on XII, resembled in size those of the plant used as rootstock, e.g., XII on XII, more than those of the plant used as scion. The dominance of rootstock influence was confirmed by McKenzie in 1956, not only for effect on growth, but also for effect on anatomical structure (26). This does not mean, of course, that the scion had no effect; but that the rootstock modified the scion more than the scion modified the rootstock.

The influence of scion stem in single-worked trees.—Work on the influence of scion stems on the growth rate of rootstocks has been hampered by the difficulty of measuring the increase in size of a root system growing in soil. Some complete excavations of mature trees on different rootstocks, made by Rogers & Vyvyan (27, 28), Beakbane & de Wet (29) and Coker (30) involved moving up to 60 tons of soil per tree.

These workers found that the roots of different Malling clones retained their own distinctive morphological characters even when growing below the same scion variety for 10 or 20 years. Scion varieties as contrasting as Lane's Prince Albert, Bramley's Seedling, and Cox's Orange Pippin did not alter the distinctive rootstock characters. Moreover, scion roots of Bramley's Seedling were remarkably different from roots of M.I, II and IX under the same Bramley's Seedling scion in 15-year-old trees (29). Another important fact found in this work (28) was that the stem:root weight ratio remained remarkably constant, for given soil fertility conditions, whether the tree was on a dwarfing rootstock, or on a very vigorous one making it more than 10 times the size of the other. Thus the concept of equal ratios of new stem and root increments, established by Knight (31) for young trees, is shown to persist for mature trees of widely varying vigour.

The difficulty of examining root systems has led many workers to base their conclusions on the behaviour of young trees, often only two or three years old. As it has been shown by Hatton (2) and confirmed by later workers, that the relative effect of different rootstocks on scion vigour may change markedly with time, it is unlikely that scion effects recorded for two- or three-year-old trees can represent, in all respects, the long-term influence of these scions on the growth of the rootstocks studied. Nevertheless, work on young trees has proved valuable for certain purposes, so long as its limitations are realised.

XUM

The early work of Swarbrick & Roberts (32) and Roberts (33, 34, 35), showed striking effects of scion on seedling root morphology, especially in the absence of rootstock stem. These authors drew the general conclusion that rootstock influence is situated in the stem rather than in the root, and that when the stem is eliminated, by grafting on roots, the rootstock is dominated by the character of the scion. Vegetatively propagated applé rootstocks have not been found to respond in the same way to scion influence, however. Vyvyan (36) found that two-year-old root systems of M.II and M.IV retained their distinctive morphological features irrespective of the variety used as scion. He also found that root systems of seedling stocks did not take on distinctive morphological characters according to the variety used as scion, whether or not a length of rootstock stem was left below the graft union. Later workers have provided some evidence in each direction. For instance Hilkenbäumer (37) while confirming effects of rootstock on scion generally similar to those found at East Malling, also quoted certain scion influences on type and form, as well as amount, of root growth.

Tupicyn (38) claimed that the morphology of the root systems of seedlings of *Prunus sogdiana* was greatly influenced by the scion type; but the morphology of his control plants also appeared very variable. Hulsmann (39) working with young apple, pear, and plum trees in the nursery found that, although the scion variety exerted a distinct influence on the quantitative development of the root system, it did not alter the characteristic morphology of the rootstock clone. This agrees with the findings quoted above that clonal rootstocks retain their distinctive morphology, whatever the scion above them, throughout the life of the tree. In fact, both the rootstock and scion variety retain their own genetic characters. Thus, the main weight of evidence is against the general conclusion that rootstock morphology is dominated by the scion, or that rootstock influence resides exclusively in the stem (19, 20, 21, 25 to 30, 36, 86, 127).

It is, of course, difficult to compare rootstock and scion influence unless the same range of clones is used both as rootstock and scion. In experiments where commercial scion varieties are grafted on to totally different rootstocks the magnitude of the rootstock and scion effects will naturally depend on the range of vigour or other characters of the particular rootstocks and scions used. In general there is a much wider range of rootstock vigour than of scion vigour, because rootstocks, and especially apple rootstocks, have been selected for vigour differences for centuries, whereas scion varieties have been chosen for characters other than vigour range.

Incidentally, Vyvyan's work (25) shows clearly that a scion can be made to grow more vigorously than it would on its own roots. The old idea that this was unlikely may have arisen because most scion varieties are inherently quite vigorous.

There is no doubt that scions can have great effects, both quantitative and to some extent qualitative, on the rootstocks below them. But where

an exact comparison has been provided by the use of the same clone as rootstock and as scion, as in the work of Vyvyan (25) and McKenzie (26), it is clear that the influence of a given clone on vigour is greater when used as a rootstock than as a scion.

The use of interstocks.—Intermediate stempieces, or interstocks, have been used both for invigoration and for dwarfing, and also for introducing resistance to winter frost and to certain diseases.

The use of interstocks as stembuilders to produce tall and vigorous trees more rapidly has been investigated by several workers including Grubb (40) who found that interstocks of three vigorous varieties had an invigorating effect. He also reported some evidence that the length of interstock affected its influence; but once again, the effect of the interstock was less than that of the rootstock.

Rogers, Beakbane & Field (41) found that the morphological characters of the root systems of M.IX and M.XII apple rootstocks were not influenced by three different intermediate stempieces 5 ft. in length, though the intermediate had some effect on the size of the root system. Seedling variation was not eliminated by uniform interstocks in trees on seedling rootstocks. Stem:root ratio was little affected by the intermediate.

Many workers, including Knight (42), Tukey & Brase (4, 43), Blair (44), Müller & Borck (45), Sax (46) and others (47, 48), have found a marked dwarfing influence from the use of dwarfing interstocks, particularly of M.IX. Blair pointed out in 1938 that dwarfing interstocks might be of commercial importance. Tukey & Brase (43) found the influence of an M.IX interstock to be less than that of an M.IX rootstock. They concluded that in many cases dwarfing could be more conveniently obtained by the choice of suitable rootstocks than by double-working. Sax (46) also found the dwarfing effect of an apomictic apple variety used as an interstock less than when it was used as a rootstock. Müller & Borck (45) confirmed that dwarfing and good anchorage could be combined by the use of an M.IX interstock on a well anchored rootstock, but reported that this advantage was more than outweighed by prolific sucker development from the roots. They also reported greater effects from a 40 cm. than from a 20 cm. length of interstock, but their growth data were very variable. The use of frost and disease-resistant frameworks is discussed later.

Sax & Dickson (49) have described the use of ring-grafting as a means of reducing growth. The most successful method was by the use of two inverted bark rings with seams on opposite sides of the trunk. The dwarfing effect was found not to be permanent because the rings became bridged by tissues formed from the original trunk.

The "fruiting bridge" (a ring of bridge grafts) used by Schulz (50) is claimed to have a more permanent effect in reducing the growth rate of apple trees than girdling or root pruning.

It should be noted that provision of an interstock by double grafting

XUM

in one operation [Garner (51)] is materially different from the two stage method normally used for stem-builders. In the former case, as also with the use of rings, the interstock normally bears no leaves; whereas in the latter the future interstock forms the only scion for a year or two, and may have a distinct influence on the rootstock during this period. From the practical point of view, the length of interstock that can economically be double-grafted in one operation appears to be limited to about 5 in., because of the risk of drying out before union has taken place.

In the interpretation of the influence of interstocks, ring-grafts, and inarches, on growth, the possible effects of virus diseases must be considered. As already mentioned, certain tree viruses have been clearly shown to have a dwarfing effect. If such a virus were introduced with an interstock, or indeed with any component of a composite tree, the interpretation of effects on growth would be made difficult, or even impossible. It seems highly probable that some of the stunting effects that have followed the use of interstocks have been caused by the introduction of virus. There is also the possibility that even where the interstock is healthy at the time of grafting it may be susceptible to a virus carried by stock or scion. Tukey, Klackle & McClintock (52) have suggested that the development in the interstock of a latent virus carried by the scion variety may have caused the severe dwarfing and other symptoms of uncongeniality that occurred in apple trees worked with interstocks of Virginia Crab. Smith (53) has suggested the possibility that the inconsistent incompatibilities of Virginia Crab reported in the literature may well be a result of the response of the "nurse roots" to a virus, and not an actual difference in the Virginia Crab or in the varieties with which they were grafted. He points out that the "wood-pitting" and the general appearance and behaviour of the abnormal trees, bear a striking resemblance to the symptoms occurring in citrus trees infected with the virus disease "quick decline."

The general conclusion appears to be that rootstock influence can reside both in the rootstock root and the rootstock stem, and that rootstock root and stem can themselves be modified to some extent by the scion; but of all these influences, that of the root is the most powerful. The use of interstocks or bark rings (apart from introduction of virus) as a growth regulatory practice can only have a limited effect, compared with that of a whole root system; but nevertheless it may be a convenient practice for certain purposes.

Rootstock and Interstock Effects on Fruiting

Rootstock effects.—The effects of dwarfing apple rootstocks on precocity of bearing and fruit set, were well established by early work [Hatton (2)]; and similar effects of roots alone, in the absence of rootstock stem, were found by Beakbane & Rogers (19). In recent years there has been an increasing interest in the effect of rootstocks on the quality, colour, storage, and

preservation properties of fruit, as well as in the quantity produced. Marsh & Cameron (54) found consistent differences due to rootstock, in bitterness of the juice of Washington Navel oranges. Navel on nearly all rootstocks gave bitter juice if the fruit was harvested early. On some rootstocks, e.g., grapefruit, the principle controlling the development of bitterness had nearly disappeared by the time the fruit was ripe for picking; on others, however, viz. trifoliate, sweet orange, sour orange, and Navel rootstocks, it only disappeared if the fruit was left on late; and on Rough Lemon rootstock it never disappeared. Kefford *et al.* (55) also found stock effects on the development of bittnerness in canned orange juice. Gurgel *et al.* (56) found that the amount of ascorbic acid in fruits of the Natal orange varied with the rootstock. The pattern of variation in ascorbic acid content throughout the season was also affected by rootstocks.

Wallace (57) reported rootstock effects on storage quality of apples. The most dwarfing rootstocks, e.g., M.IX, tended to produce fruits which stored least well, and also were highest in reducing sugars. The work of Kidd & West (58) has shown, however, that the effect of rootstocks on the storage of apples is not a simple one and varies according to manurial treatment and other factors. It has been found that apples from trees on M.IX can be stored satisfactorily provided they are picked at the right stage of maturity; this entails picking them earlier than fruit from trees on more vigorous rootstocks. Phillips & Nelson (59) also found rootstock effects on storage of apples.

Other fruit characteristics, such as size and colour in apples (60), red colour in blood oranges (61) and size, colour and flavour in red currants (62) have been shown to vary according to the rootstock used.

Interstock effects.—Hewetson (63) found that out of 10 interstocks, M.IX had a marked effect in hastening the maturity of the fruit and increasing its colour. Yields were increased by the variety Haas and reduced by the variety Dudley used as interstocks. Blair, Cannon & Beingessner (64) reported an influence of interstock frames of M.IX and M.IV on precocity of bearing. Longley (65) found that Virginia Crab frames induced precocity in some varieties, but the later development of the trees was unsatisfactory. Abrahamse (66) reported that the yield of pear varieties was affected by the interstock used.

Thus it is clear that interstocks can affect precocity of bearing and quality of fruit; and, at least so far as M.IX is concerned, the effects tend in the same direction as those of an M.IX rootstock.

Stock/Scion Incompatibility

In their reviews of work on stock/scion incompatibility, i.e., the failure of certain combinations of plants to intergraft, Argles (8) and Herrero (67) listed several factors that had been considered by earlier workers to affect compatibility. Some of the hypotheses based on these factors have not been supported by later work, as, for example, those concerning differences in

XUM

season of growth, in vigour, or in rate of callusing. One, however, has been extensively developed, viz. that of metabolic differences between stock and scion. It has also become apparent that virus diseases may be responsible for some stock/scion failures that have previously been attributed to incompatibility, one of the earliest records in apples being that described by Shaw & Southwick (68) between the clonal rootstock Spy 227 and certain scion varieties.

De Stigter (69), in a comprehensive study of incompatibility in cucurbitaceous grafts, concluded that this was caused by factors genetically inherent in the graft partners, rather than by a virus disease. He showed that for melon to make healthy growth when grafted on *Curcurbita ficifolia*, it was necessary to allow some foliage to develop on the stock, and to maintain a balance between the size of the melon scion and the number of stock leaves. Removal of the stock leaves at any stage resulted in death of the plant. He concludes that an active principle emanating from the stock leaves, functions primarily in keeping the sieve tubes in a condition in which enzymatic processes can take place in the normal way. Changes were recorded in the relative amounts of starch in the stock and scion with changing numbers of stock leaves. These phenomena were tentatively assumed to result from the interaction of the photosynthesizing capacity of the melon top, and the carbohydrate mobilizing capacity of the stock leaves.

By contrast, Mosse & Garner (70) found that the combination Victoria /President/Myrobalan B plum showed incompatibility, although both Victoria and President are compatible with Myrobalan B. Allowing the interstock to bear leaves did not lessen the incompatibility, so a different mechanism appears to be involved here.

De Stigter (69) also tested intermediate stempieces of melon between scions of cucumber and stocks of *C. ficifolia*. A long intermediate caused incompatibility; but when the intermediate was extremely short ($1\frac{1}{2}$ mm.) the same combination remained healthy, even after removal of the stock leaves. De Stigter concluded that the composition of the stream of metabolites was changed during transport through "foreign" tissue, and that the changes were reduced in extent when the intermediate was very short. He suggests that some substance required by *C. ficifolia* is either lacking in the melon, or is present in an inactive form, rather than that the melon produces a substance acting as an enzyme inhibitor in the stock.

An accumulation of assimilates in the scions of incompatible combinations was noted by Friedrich with apples (71), Herrero with cherries (67), and Boubals & Huglin with vines (72). These workers concluded that substances elaborated in the scion were unable to pass the incompatible union. Another possible interpretation is that of de Stigter, who suggested that elaborated substances may be transported in some simpler form and that recombination may depend on the presence of a suitable enzyme in the stock. Such an enzyme may be lacking in incompatible combinations.

Herrero (67) noted two different types of incompatibility in plums, pears, and peaches. In one type, a layer of undifferentiated tissue at the union caused mechanical weakness, but the growth of the trees was normal, often for many years; and in the other, the trees showed early symptoms of ill health, with premature degeneration of the phloem. He concluded that abnormalities at the union were not the direct cause of incompatibility, however. Double-grafting with a compatible intermediate, so widely successful in overcoming incompatibility for pears on quince (51), has been shown to be ineffective for plums [Herrero (73)], and for apples [Sax (74)].

The relative positions of the graft partners in double-grafted trees has been shown to affect compatibility. Mosse & Herrero (75) found that ring-grafted trees of Conference pear on Quince A with a ring of the pear C8 inserted in the rootstock stem were more incompatible than double-grafted trees of Conference on Quince A with an intermediate stempiece of the pear C8. The degree of incompatibility depended on the relative positions of C8 and QA. Wherever any tissue of QA occurred above that of C8 the trees grew poorly whereas the combination Conference/C8/QA grew well. It appears, therefore, that here the incompatibility was not the result of an uncongenial leaf system, but was possibly associated with the polar movement of some substance that adversely affects the tissues of the pear C8.

Methods of overcoming incompatibility.—The generally accepted method of overcoming incompatibility in pears on quince is by the use of an intermediate stempiece compatible with both scion and rootstock. When this practice was first adopted, it was usual to insert, as a graft, a length of several inches of intermediate stem. Garner (76) has shown, however, that an extremely small piece of a mutually compatible variety may serve to bring about a sound union between a pear and quince normally incompatible; and he has described a double-budding technique in which the scion bud-shield is supplemented at its base by a small shield of an intermediate variety without any bud of its own. A slightly different form of double-shield budding has been described by Nicolin (77).

Thiel (78) suggested that the degree of compatibility of two plants depended on their ability to reabsorb the primary isolating layer formed between stock and scion. She presented evidence that the action of the supplementary shield in double-shield budding in overcoming incompatibility is largely due to a strengthening of the ability to reabsorb this layer.

Forecasting compatibility.—A feature of incompatibility is that it does not always produce visible symptoms in growth, or leaf characters, in young trees. In fact, recognition of the trouble in the field is often not possible for periods of up to ten years with some combinations of plum, and of pear on quince. In one instance, with walnuts, incompatibility did not show until the trees were 40 years old [Garavel (79)]. Passecker (80) suggests that some forms of incompatibility may increase with the age of the plant.

In an attempt to develop a rapid laboratory method of forecasting the

XUM

compatibility of different clones, to avoid prolonged trials in the field, Herrero (67) examined the histological structure of the graft partners in plum and pear trees differing in their degree of compatibility. He found no correlation between compatibility and stem structure of the graft partners; but he established that abnormalities were present in the structure of the unions of one-year-old trees of incompatible combinations of plum and of pear on quince. These abnormalities were visible under the microscope before any effects on growth could be seen; thus he showed that the structure of the union in young plum and pear trees can be used to forecast incompatibility. He suggested that the fundamental causes of this trouble reside in the metabolic reactions of the neighbouring cells of stock and scion close to the point of union.

The possibility of special graft affinities, or a synergistic effect of certain combinations when compared with the same components ungrafted, is indicated by Kirchhoff's work on red currants (81). But this invigorating effect was not fully maintained three years later (61). Another indication of graft affinity is provided by Sax (82). He described the failure of the progeny of species hybrids, and occasionally of pure species, to grow satisfactorily on their own roots, and reported that these plants sometimes grew well when grafted on suitable rootstocks. Examples are given from *Syringa* and *Malus*.

Other Features

Resistance to pests and diseases.—An example of successful breeding to combine a range of rootstock vigour with resistance to an insect, is provided by the new Malling-Merton range of woolly-aphid resistant apple rootstocks (83, 84). Four of these, namely Malling-Merton 106 (semi-dwarfing) and MM.104, MM.109, and MM.111 (vigorous) are already widely planted in England. The resistance applies to the rootstock, not the scion; but it eliminates the woolly aphid from the stoolbed, and prevents the development of the root form of it in the plantation. An encouraging feature of this work is that from less than 4000 seedlings at least 10 clones with the desired combinations of features have been obtained. This work took some 25 years, but with the new quick methods of selection for vigour and other features (85, 86) much quicker results would be likely in future.

Thus so long as a parent plant is available, containing any particular feature which it is desired to incorporate in a rootstock, but lacking other good features, it seems likely that the desired combinations of characters could be incorporated in new ranges of rootstocks, by breeding. Breeding rootstocks is simpler than breeding scion varieties, because no fruit characters are involved. It is clear that only the fringe of possible advances from rootstock breeding has yet been touched; for instance, breeding for resistance to Collar Rot (*Phytophthora cactorum*) in rootstocks should be a fruitful line of work.

The use of resistant frameworks (stems and crotches) of Myrobalan B

and certain other plums against bacterial canker *Pseudomonas mors-prunorum* Worm. in plums was shown by Montgomery *et al.* (87) to be a useful defence against this disease, and similarly Grubb (88) showed that cherry trees high worked on the Mazzard F12/1 were less damaged by bacterial canker than low-worked trees.

Resistant forms of French pear, which produce seedlings resistant to fire blight (*Erwinia amylovora*) for use as rootstocks, have been developed by Reimer (89). The mahaleb rootstock is reported as useful for cherries in California because of its resistance to the buckskin virus, and also because, if infected, it dies quickly without remaining a source of infection [Rawlins & Thomas (90); Day (91)]. It is also recommended in Utah, as resistant to Western X virus [Richards & Cochran (92, p. 59)].

Resistance to winter frost.—A wide range of resistance to winter cold exists within apple rootstocks and scion varieties. Current information was reviewed by Kemmer & Schulz in 1955 (93). The species *Malus baccata* and the varieties Antonovka and Hibernal have been found especially resistant to winter injury [Oldén (94); Wilkinson (95)].

Several varieties, for instance Antonovka and certain French crabs have proved useful as interstocks, to give hardy frameworks [Filinger (96); Filinger & Zeiger (97)], and the value of including a whole branch of a hardy variety in the frame of a tender variety has been emphasized by Filewicz & Modlibowska (98).

The Malling rootstocks were not chosen for frost hardiness, but in spite of this they are used successfully in most parts of Western Europe including areas which experience winter temperatures below zero Fahrenheit. Malling XI, M.XVI and Alnarp 2 have proved especially resistant in severe winters [Oldén (99); Karnatz (100); Athenstädt *et al.* (101); and others]. Some variation in reports of rootstock susceptibility can be explained by the observed connection between dormancy and hardiness. Thus M.XVI, breaking its dormancy late, proves resistant to late winter frost; but since it matures late in the autumn it is susceptible to early winter frost. Malling IX, which is early leafing and early maturing, is relatively resistant to early winter frost, but susceptible to late winter frost.

Schultz & Graves (102) reported that the hardiness of the Malling stocks was increased by the use of Dolgo crab, an exceptionally hardy variety, as the scion; but such an effect is by no means universal [Filewicz & Modlibowska (103)]. For some fruits, rootstocks belonging to a different genus from that of the scion are used as, for example, *Cotoneaster lucida*, *C. melanocarpa*, and *Chaenomeles japonica* for pears [Kelli (104)]. Frost resistant frames are obtained for mandarin oranges by top-working on to a resistant variety [Prohorov (105)].

In citrus there appears to be a rootstock effect on the hardiness of scion varieties [Cooper (106)]. Such an effect has not been recorded for pome fruits.

XUM

The value of frost-resistant rootstocks and frames in severe climates is well established, however; breeding and selection for winter hardiness is continuing at several centres including Balsgård, Ottawa, Morden (Manitoba) and in Russia. Rapid tests for determining hardiness by electrical conductivity methods have been reported successful, and may help plant breeders and selectors (97, 107).

Root anchorage.—The great interest in control of vigour and precocity by rootstocks has tended to divert attention from that important function of the root, anchoring the tree in the soil. Apple rootstocks show a wide range of anchorage, and two widely used ones, M.IX and M.IV are weak in this respect, and even M.II is only fair, although M.XVI is excellent [Preston (108)]. Several of the new Malling-Merton rootstocks are better anchored than comparable ones of the Malling series; for instance, MM.104 is much better anchored than M.IV and MM.111 than M.II (108). Some of the reasons for poor anchorage e.g., short fibres, can readily be seen by microscopic examination and here again the selection of well-anchored new rootstocks by quick methods seems feasible [cf. Beakbane (86)].

In general the dwarf rootstocks tend to be brittle rooted. The use of a dwarfing interstock on a well anchored rootstock, to give a well anchored dwarf tree has been suggested by several writers, as mentioned above; and in spite of difficulties this appears to be worth further trial. As fruit trees are commonly staked, in Europe, for the first four to seven years, the elimination of this labour and expense would be a useful advance.

The above sections on anchorage, and on resistance to pests, diseases and frost, mainly contain examples of the direct use of the characters of the rootstock or interstock itself, rather than their effect upon the scion characters. By contrast the next three sections are examples of rootstock effects upon the scions.

Resistance to spray damage.—Preston, Moore & Bennett (109) report a rootstock effect on the amount of lime sulphur spray damage occurring on dwarf pyramid apple trees. In general trees on dwarfing rootstocks were less damaged than those on vigorous ones, but there were exceptions.

Rootstock influence on rooting of cuttings.—Garner & Hatcher (110) have recently shown that hardwood cuttings taken from hedges of Crab C apple grafted on vigorous rootstocks, rooted much better than cuttings from similar hedges grafted on dwarfing rootstocks. This is a clear indication of a change in physiological conditions of the scion, due to rootstock influence, and probably associated with vegetative vigour of the shoots.

A method of rooting difficult cuttings of pome and stone fruits described by Kirchhoff (111), involved inarching the tops of the cuttings into nurse rootstocks, which helped to keep them alive until they had rooted, after which they were severed from the nurse plant. In this instance different rootstocks did not appear to affect the rooting.

Increased range of rootstock effects.—Another series of apple rootstocks

bred at East Malling has greatly extended the range of vigour of rootstocks. These are the M.IX Crosses, so called because M.IX was one of the parents in each case [Tydeman (112)] and they include rootstocks much more dwarfing than M.IX, and some more vigorous than M.XVI. The ratios of trunk cross sectional area between Cox's Orange Pippin on M.IX and on M.XVI at seven years old was about 1:4; whereas that between trees on 3426 and 3430, was about 1:15 [Preston (113, 114)]. These rootstocks have not yet been released, and many of them never will be, because they sucker too much (113). But they are of great scientific interest, and some may usefully supplement the Malling rootstock range. This forms another example of the practicability of improving rootstocks by breeding.

The present paper has dealt mainly with rootstock effects on pome and stone fruits, with which the most progress has been made; but it should not be forgotten that rootstock effects have been used in many other crops (11). Rootstock effects are of great importance in citrus (115, 54, 55, 56, 121 and many others), and are also used, for example, in vines (116), roses (117), walnuts (118), mango (119), tung (120), and many others. It is outside the scope of this paper to deal with these crops, but it is likely that many of the same fundamental principles will apply to all.

Hypothetical Mechanisms of Stock/Scion Interaction

There appear to be three principal ways in which the genetically distinct clones used as parts of a composite tree may affect the growth of the tree as a whole. They may differ (*a*) in their capabilities for uptake, synthesis, and utilization of nutrients, (*b*) in their capacity for transporting nutrients, and (*c*) in the type and quantity of plant growth regulating substances present in the tissues.

Nutrients.—Wallace *et al.* (121) have suggested that root respiration may be a measure of the capacity of citrus roots for mineral uptake, and they have shown that the mineral composition of the scion foliage may be influenced by the rootstock. Ruck & Bolas (122) have shown that varietal differences in net assimilation rate occur in the apple. The rates for four rootstocks grown under a wide range of cultural conditions were in the order of vigour of the stocks, being higher in vigorous than in dwarfing stocks. A rootstock effect on the net assimilation rate of an apple scion has also been demonstrated, the variety Cox's Orange Pippin on vigorous rootstocks having a higher rate, over the whole season, than on dwarfing rootstocks [Gregory 123)]. This difference arose largely because photosynthetic activity continued much later in the season on vigorous than on dwarfing rootstocks.

The rôle of leaves as organs in which compounds are synthesized was understood long before the importance of roots in synthesis was recognized. During recent years, however, attention has been directed towards the metabolism of roots. For example, it has been proved that nicotine occurring in leaves of tobacco, or in leaves of tomato grafted on tobacco, is formed in

XUM

the plant's roots [Dawson (124)]. Bollard (125) has stated that, in apples, inorganic nitrogen is largely converted into organic form in the root system. The part played by the root in synthesis would fit in with the observation that by no means all of the effects observable from a complete rootstock can be obtained from an interstock even of great length.

In composite plants, the tissues of a variety may contain, and possibly its growth may be affected by, organic compounds that would not be present in ungrafted plants of the variety. Although it has been shown that, in some instances, characteristic elaborated compounds formed by the graft partners may not pass the graft union [Williams (126)], in others they clearly do so [Dawson (124)].

The importance of parenchymatous tissue in the economy of the plant is evident from the fact that it is the tissue mainly concerned with the functions of respiration and storage, and thus with the utilization of nutrients. Varietal differences in amount and distribution of parenchymatous tissue have been recorded in apple. In addition to a high bark:wood ratio, dwarfing rootstocks have been found to possess a highly parenchymatous xylem and phloem, and thus to contain more living tissue per unit volume of stem and root than vigorous rootstocks [Beakbane and co-workers (127 to 131); Carlone (132)]. The metabolic requirements of roots must, therefore, differ in different rootstocks, being greater per unit volume of tissue in dwarfing than in vigorous stocks. Thus while the stem:root ratio in apple trees of different vigour is about the same a much greater proportion of the root system is composed of living tissue in dwarf than in vigorous trees (28, 86). It is therefore clear that competition between sites at which carbohydrates are utilized will be likely to occur, and that the roots will utilize a relatively greater proportion of the total assimilates in dwarf than in vigorous trees. The size of this difference in the proportion of carbohydrates utilized by the roots may, moreover, be modified to some extent by differences in rate of respiration. It has been suggested (133) that, in dwarfing rootstocks, a large living tissue to plant surface ratio may result in a deficiency of oxygen leading to slower respiration, while in vigorous rootstocks a small living tissue to plant surface ratio may result in a plentiful supply of oxygen leading to more rapid respiration. Hassan (134) found that the rate of respiration per unit of living tissue was higher in vigorous than in dwarfing rootstocks.

Thus it is clear that different clones may differ greatly in their capacity for absorbing, storing and utilising nutrients.

Transport.—It has been suggested that the movement of metabolites in the phloem may depend, to some extent, on the size of the sieve tubes, and that the relative capacity of the phloem for conduction and storage may be an important factor in the control of growth (133). Any sudden decrease in sieve tube size or number, occurring at the union between two graft partners, might severely check the downward flow of organic nutrients and lead to an accumulation of carbohydrates in the scion. Dickson & Samuels (135), study-

ing the mechanism of dwarfing effect in apples with the aid of radioactive phosphorus, concluded that the great concentration of the isotope found in dwarfing interstocks, or in inverted rings of bark, indicated that the dwarfing effect of these intermediate stempieces may have been caused by a check to the flow of organic nutrients to the roots.

The structure of the xylem, the principal water-conducting tissue of plants, has been found to differ in apple rootstocks and scion varieties of different vigour [McKenzie (26); Beakbane (86, 128, 133); Beakbane & Renwick (127); Beakbane & Thompson (129, 130); Thompson (131)]. In general, vigorous rootstocks, and strongly growing scion varieties, have been found to possess large vessels. The greater size of the vessels in vigorous plants together with their greater frequency may result in up to 25 per cent of the xylem being composed of vessel tissue in roots two to three years old, whereas in roots of the same age from dwarf plants, as little as 5 per cent of the xylem tissue may be composed of vessels. These differences in the sizes and numbers of vessels are sufficient to suggest that the capacity of the xylem for water transport would be higher in vigorous than in dwarf plants; but this may not be a limiting factor. Knight (136) working with detached stems of apple rootstocks of differing vigour, found no great differences in water translocation under pressure. Preston (137) expressed the view that the number of vessels in stems is normally greatly in excess of that required for transport of water. On the whole it seems more likely that phloem transport, rather than xylem transport, would be a limiting factor in rootstocks and interstocks, and this agrees with the powerful effects of inverted bark rings, and phloem-blocking viruses.

Growth regulating substances.—From an extensive series of studies at East Malling, on rootstock:scion interactions, McKenzie (26) has put forward a tentative hypothesis that growth inhibiting substances may play an important part in determining the vigour of apple clones. He reached this conclusion, not from growth substance measurements, but by analogy with the performance of other plants in which such measurements have been made. This hypothesis is in line with the work of van Overbeek (138) who found that a dwarf variety of corn contained a much lower auxin level than certain more vigorous varieties. The effect of low auxin levels was shown to be due, not to a lower auxin production rate, but to a high rate of auxin destruction. The dwarf corn had a more branching habit than the vigorous varieties and also showed a tendency to become prostrate. It may be noted that one of the characteristic features of dwarfing apple rootstocks is their branching habit, the extremely dwarf types, such as 3426 for example, having a definite tendency to become prostrate when allowed to develop their natural form. Delisle (139) has shown that the production of relatively large amounts of auxin in the apex is associated with the development of a relatively unbranched form in certain aster species, and conversely plants that contain low auxin levels develop a branching habit.

XUM

Thus it appears that the type of growth induced by dwarfing rootstocks is similar in many ways to that of plants in which it has been shown that the auxin level is low. A theory depending, not on the production of auxin in the scion, but on its destruction or inhibition by substances in or coming from the stock, would fit many of the observed rootstock and interstock effects; but no direct experimental evidence is yet available.

The effects of complete root systems could be accounted for by all three of these postulated mechanisms, viz. differences in nutrient absorption and metabolism, in transport, and in auxin metabolism; but the effects of interstocks, rings, and rootstock stems could presumably only be produced by the second and third of these mechanisms. It is probable, however, that no one of them operates alone, and that all work together.

It is fashionable, in writing of rootstocks, to quote that rootstock effects were known and used by the ancients thousands of years ago. True as this is, and much as our knowledge has increased, it is clear that our understanding of stock/scion relations is still only imperfect. The field is wide open for further great advances, both practical and fundamental.

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ASCENT OF SAP[1,2]

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Introduction

The problem of the ascent of sap in plants, particularly in tall trees, has invited the attention of botanists and others for many years. However, despite this long history of investigation, numerous aspects of the phenomenon remain to be clarified. Concern with the problem approached a climax in the late nineteenth century with the presentation by Dixon & Joly (1) and Askenasy (2, 3) of the cohesion or liquid tension concepts of the ascent of sap. Subsequently, however, interest in the actual process of water conduction through the standing plant has diminished, although such related phenomena as water absorption and the water relations of cells and tissues have remained areas of very active investigation.

The literature of moisture movement in plants is very extensive, a consideration which necessitates a somewhat arbitrary choice of material for review purposes. Thus this essay deals largely with physical aspects of the process of water conduction in large woody stems, with particular emphasis on the suggested mechanisms of transport. Attention has been focused upon the more recent literature of the subject; for extended considerations of the problem covering progress up to the early twentieth century, reference may be made to the works of Darwin *et al.* (4), Pfeffer (5), Strasburger (6, 7), Copeland (8), and Dixon (9, 10).

Prior to 1890 several hypotheses had been advanced in explanation of the ascent of sap in plants. Physical as well as physiological mechanisms had been envisaged, including a pumping action by living cells, imbibition and movement through cell walls, capillarity, gas pressure, air pressure, and liquid distillation (8 to 11). However, subsequent analysis and experimentation revealed apparent shortcomings in practically all the suggested schemes (9, 10, 11). The most widely accepted of current generalizations was advanced in 1894 when Dixon & Joly (1) published a preliminary account of a cohesion mechanism in explanation of the upward transfer of water. This was followed by two papers by Askenasy (2, 3) who postulated a mechanism somewhat similar in conception to that envisaged by Dixon & Joly. In the succeeding 30 years the concept was much extended and amplified by the studies of Dixon and associates (12 to 15), Renner (16), Bode (17), Holle (18) and Ursprung (19), and more recently by Smith *et al.* (20), Hungate (21), Baker & James (22), Strugger (23), and many others. Crafts *et al.* (24) have recently attempted a further refinement of the concept through a

[1] The survey of literature pertaining to this review was concluded in August, 1956.

[2] Contribution No. 334 from the Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

XUM

preliminary analysis of the interrelations between sorption phenomena, micro-structural considerations, and the physical properties of water. In view of its wide acceptance the cohesion mechanism is treated at some length below. Consideration of recent, alternative mechanisms follows in later paragraphs.

Cohesion Theory of Ascent of Sap

According to the cohesion hypothesis, moisture is lost through evaporation from the microcapillaries of the walls of the parenchyma cells of the leaf. Physical forces in turn lead to the movement of moisture from protoplasm and vacuole to the cell wall. This movement results in an increase in the osmotic concentration of the cell, and water is withdrawn from adjoining cells. [For recent discussions of the interrelationships between physical factors causing this transfer of moisture, reference may be made to the works of Homan *et al.* (25), Smith *et al.* (20), Shull (26), Kramer (27), and Levitt (28)]. Eventually a gradient extends to the contents of the xylem elements of the leaf and ultimately to the water of the conducting and perhaps other elements of stem and root.

The cohesion hypothesis assumes continuity of water columns, laterally and vertically, in the conducting elements of the xylem. These water columns ultimately are placed under tensile strain, but widespread rupture is believed not to occur owing to the purported cohesive properties of water when entrapped in small capillaries. As a consequence of the pull resulting from the loss of moisture from the leaves, water is drawn up the stem from roots to foliar surfaces.

Notwithstanding its several assumptions, the liquid tension hypothesis appears to meet numerous structural and functional requirements with respect to moisture movement in plants, particularly in those of low stature (29), and the mechanism has gained wide acceptance amongst botanists (23, 24, 30, 31, 32). As intimated above, the proposals advanced by Dixon & Joly and Askenasy prompted immediate and widespread interest, and stimulated much experimental work designed to test, amplify, or clarify specific aspects of the cohesion mechanism. Of first importance in this regard are the numerous and significant investigations of the tensile properties of water.

Tensile strength of water.—Values of the cohesive strength of water to be derived from theoretical considerations are very high. Surface tension relationships (33), Laplace's theory of capillarity (34), internal pressure measurements, and heats of vaporization lead to predicted values of the order of 15,000 atm. However, experimental attempts to demonstrate a high tensile strength have led to conflicting results. Wide discrepancies are evident in the values obtained by different workers employing a particular technique, and in the results gained under differing experimental conditions, statical, acoustical, and dynamical.

Repetition of the work of Berthelot led Dixon (10) to suggest that water

entrapped in glass tubes of small diameter may withstand tensions exceeding 200 atm. without fracture, and to contend that this property is not affected significantly by the presence of dissolved air. Renner (16) and Ursprung (19), utilizing biological materials, concluded that water in fern annuli may support tensions of the order of 300 atm. prior to fracture. Recently, Haider (35) has advanced estimates of the same magnitude. Similarly, King (36) as a consequence of biophysical studies with fern annuli has concluded that the tensile strength of water is of the order of 200 atm. However, it has been shown that his data are consistent both with lower or higher values (37). In addition, King (36) has reported that Haller, with the aid of a piezo-electric device placed in a stream of water wherein cavitation occurred, obtained a value of 200 to 300 atm. for the tensile strength of water. Investigations by Kenrick *et al.* (38) on the superheating and saturation of liquids suggest a value of approximately 100 atm. for the tensile strength of water. Harvey *et al.* (39) found that water may withstand large tensile stresses if the liquid is afforded a very rigorous pretreatment consisting of the application of very high positive pressures for long periods to remove gas nuclei. Briggs (40) employing a centrifugal method obtained values of the order of 220 atm. for the tensile strength of water.

In contrast to the foregoing rather large values, a number of investigators have demonstrated that water may have a relatively low tensile strength. Budgett (41) found that the pull required to separate flat steel plates wrung together and separated by a film of water ranged from 4 to 60 atm. Meyer (42) utilizing an improved but somewhat similar procedure to that employed by Berthelot, obtained a value of 34 atm. for the tensile strength of water. Temperely & Chambers (37) and Temperely (43) made a thorough analysis of the Berthelot method, and obtained an average tensile strength of 32 atm. Similarly, Scott *et al.* (44) undertook a painstaking re-examination of the Berthelot technique, and reported a value of 30 atm. for water under ideal conditions. In addition, Vincent & Simmonds (45), Temperely (43), and Scott *et al.* (44) have drawn attention to an apparently false assumption made by Dixon which has affected the latter's calculations by a factor of 5 to 7. These investigators have shown that contrary to Dixon's belief the pressure in the glass capillaries at the sealing temperature is not zero, but rather of the order of 100 atm., and they conclude that the very high values obtained by Dixon are the result of the higher pressures required to fill the glass tubes in the presence of small bubbles of entrapped air. Similarly, Santamaria & Loomis (46) have suggested that Ursprung's (19) values of the tensile strength of water are open to serious question because of a probable confusion of adsorption forces with cohesion. Scholander *et al.* (29), through centrifugation in glass tubes, have derived values in excess of 17 atm. for the tensile strength of water, but observed much lower values (1 to 3 atm.) when the experiments were repeated using plant materials. Other techniques employed in meas-

XUM

uring the tensile strength of liquids, and employing either hydraulic, dynamic, or acoustical procedures yield values ranging from 0.05 to 10 atm.

The foregoing observations indicate that water is able to withstand negative pressures of commensurable magnitude without fracture under suitable experimental conditions. Whether or not these tensions may reach sufficient magnitude in the lumina of conducting elements to support Dixon's conception of the functioning of the cohesion mechanism remains an open question (29). The spectrum of opinion in this regard is bounded on the one hand by the views of MacDougal *et al.* (47) who concluded that tensions as high as 200 atm. may be developed in transpiring plants. On the other hand, nucleation considerations have prompted Silver (48) to infer that the tensile strength of water is negligible under ordinary circumstances. Recent experiments in nonbiological systems suggest an upper limit of the order of 30 atm. for the tensile strength of water under static conditions and under extremely favourable experimental circumstances. The above figure is probably adequate for the proposed functioning of the cohesion mechanism (9). However, under less ideal static and under dynamic conditions, rupture at much smaller values appears to be a distinct possibility. Circumstantial evidence of widespread fracture of stretched water columns may be derived from a study of seasonal decreases in the moisture content of the wood of transpiring trees (30, 49), from injection experiments (50), and from biophysical analyses (51). Each of these lines of investigation indicates a high percentage of gas-filled, nonfunctional elements under field conditions. In this regard see also Scholander *et al.* (29), Preston (52), Boyd (53), Lundegardh (54), Greenidge (55), Lutz (56), and Day (57).

It is evident from the foregoing that experimentally-determined estimates of the tensile strength of water, and the predicted or theoretical figures based upon cohesive properties and internal pressure considerations differ very markedly. Similarly, a wide variation is apparent in the values gained under differing experimental conditions. In attempting to harmonize these wide discrepancies, appeal may be made, with profit, to recent studies of the liquid state. These analyses have given rise to a number of alternative, perhaps more satisfactory, explanations for the fracture of stretched water columns than the failure of tensile forces. Several of these possibilities are briefly described below.

It has been indicated that a liquid subject to negative pressure or tensile stress is in a metastable state (58, 59). Furth (60) has concluded that the ultimate tension which water may withstand at the limit of the metastable region is of the order of 4000 atm. His analysis suggests that rupture will occur, under ideal conditions, at negative pressures below this figure, not as a result of failure under tensile forces, but as a consequence of the formation of a vapour bubble of critical size. This finding is completely in harmony with the results of Döring's analysis (61).

Similarly, Fisher (62) has indicated that water immediately prior to collapse as a consequence of negative pressures is in a quasi-stable state.

From a consideration of nucleation and bubble formation, Fisher has concluded that under ideal conditions the pressure required to initiate fracture in water is of the order of −1350 atm. This figure is based upon the assumption that the bubble causing rupture arises in the interior of the liquid. In an attempt to bring theoretical and experimental estimates of the fracture pressures of liquids into closer agreement, Fisher (62) has examined the influence of surfaces on this calculated value. He found that in the case of a paraffin wax-water interface (contact angle 105°) the theoretical pressure is reduced to approximately 60 per cent of that required for failure in the body of the liquid. Fisher also points out that as a consequence of various surface effects, experimental pressures numerically much smaller than those noted above are to be anticipated. This suggestion is of particular significance to the present discussion in view of the probable presence of gas nuclei and other materials in the transpiration stream, and the incidence of various types of cell wall sculpture.

Temperely (63) has re-investigated the theoretical tensile properties of water through a refined analysis of van der Waals' considerations. He points out that only in the region of the absolute zero could the tensile strength of a liquid be comparable with the internal pressure. Temperely suggests that at temperatures below 270°C. tensile strength may be considered a function of temperature, the value, for water, decreasing from 800 atm. to 450 atm. over the range 0° to 100°C. He notes that while there is no experimental evidence of the existence of tensions of this magnitude, the discrepancy between theoretical and experimental figures is, nevertheless, much smaller than is commonly believed.

Thus, re-examination of the mechanism of failure and re-investigation of the magnitude of negative pressures which water may withstand without fracture have led to a partial explanation of the divergence between experimental and predicted values of the tensile strength of water. Still awaiting consideration, however, with regard to the proposed functioning of the cohesion mechanism, is the significance of the observed variation in tensile strength under differing experimental conditions.

Continuity of water columns.—The existence and maintenance of continuous columns of water under field conditions is prerequisite to the proposed functioning of the cohesion mechanism. In addition it must be demonstrated that these columns are under negative pressure during periods of active transpiration. The critical importance of these two conditions to the functioning of the cohesion mechanism has prompted much experimental work but the results obtained have proven somewhat contradictory.

Among the earlier workers, Sachs, Strasburger, Vesque, Schwendener, and others, have reported the presence of bubbles in water-conducting elements, and Copeland (8) has discussed the significance of these observations within the framework of the cohesion mechanism. Similarly, Pfeffer (5) has reported that the conducting elements of transpiring plants are occupied by a chain of water columns and air bubbles, and has speculated

XUM

upon the manner in which moisture moves upwards under these conditions. Ewart (64) has commented upon the existence of bubbles in vessels and tracheids, and questions the validity of concepts of moisture movement which fail to take into account the occurrence of these discontinuities in the contents of conducting elements. Alternatively, Bode (17) has criticized, on methodological grounds, certain of Strasburger's observations dealing with the occurrence of gases in conducting elements. In company with Holle (18) he subjected conducting tissue to direct microscopic examination, and reported the existence of continuous columns of water. Peirce (50) has suggested that water exists in the form of hollow cylinders in the smaller vessels and tracheids rather than as solid columns during periods of rapid moisture movement, and he questions the justification of applying the results of Bode's laboratory experiments to the situation existing in the transpiring tree under field conditions. Priestley (65) has concluded from injection experiments in ring porous and diffuse porous stems that vessels contain gases at low pressures, mainly water vapour. The observed simultaneous upward and downward movement of injected materials in transpiring plants (15, 66) led Preston (51) to question the existence therein of moving continuous water columns. Scholander *et al.* (29), through appeal to pressure gradient data, have obtained evidence of continuous water columns in tall vines prior to leaf flush. However, in transpiring plants during the daylight hours, no gradient was evident, the trend being reversed on occasion. Data of the same nature presented by these authors may be indicative of a water replenishment, during the night, of vessels evacuated by daytime transpiration effects, but not necessarily of a reestablishment of continuous water columns. In company with Priestley (65), Scholander *et al.* (29) have invited attention to the difficulties attendant upon the maintenance of continuous water columns in transpiring trees exposed to the wind, in view of the "great delicacy required in the laboratory to produce let alone handle tensions" much smaller than those required to raise water to the leaves of trees approaching heights of 100 m. Scholander *et al.* have concluded that there are no data indicative of the existence in tall trees of sap columns at the postulated pressures of -20 to -100 atm., and state that evidence gained from their work with grapevines is of a contrary nature.

Conversely, as noted above, the observations of Bode (17) and Holle (18) are suggestive of the existence of continuous columns of water, at least in some plants or in certain organs of plants. Haines (67) believed alterations in the appearance of the secondary xylem of transpiring trees, following insertion of a knife blade, to be attributable to mechanical rupture of continuous water columns and a subsequent change in the relative proportions of gas and water in the stem. His data also indicate an increasing number of nonfunctional elements over the growing season. Moisture distribution patterns in the wood of several species of forest trees led Gibbs (30) to postulate the existence of continuous water columns in the stems of large

woody plants. Similarly, Gibbs (30) suggested that the findings of MacDougal and associates (47) point to the same conclusion. Preston (51) found evidence of some continuous columns of water in ash stems, but concluded that the bulk of the vessels are gas filled and nonfunctional. According to Lundegardh (54), that fraction of the water of the stem which serves as a conductor of the transpiration stream forms "continuous strings from roots to leaves and is located in the medium-sized tracheids." Recently Crafts *et al.* (24) have invited attention to the ease with which bubbles may arise in the conducting elements of plants, and suggest that the discontinuities reported by earlier workers may be the result of the treatment afforded the experimental material [see also Dixon (9)].

While not proof of the existence of continuous columns of water, certain experimental data gathered in recent years are strongly suggestive of this state of affairs. The very high rates of moisture movement observed by Coster (68), Huber and associates (69, 70), and more recently Beckman *et al.* (71) are difficult to interpret otherwise than in terms of continuous columns. Huber (72) has suggested recently that the measured rates of movement are intelligible only when the vessels themselves are water-filled and serve as the conducting channels.

As noted previously, several lines of evidence point to the presence of considerable volumes of gas in the wood of actively transpiring trees. The threat posed by this gas to the maintenance of continuous water columns and to the continued functioning of the cohesion mechanism has been discussed on several occasions (4, 8, 30, 31). It would appear, a priori, that gas would diffuse readily through the wet walls of conducting elements and into the putative stretched water columns during periods of tensile strain, resulting in rupture (subsequent to the formation of a vapour bubble of critical size) and the elimination of a particular element from the conducting system. However, Dixon (9, 15) and later workers envisage the existence of a gas-water meshwork in the stem of a transpiring plant, suggesting that the purported valve-like activity of the torus of the bordered pit constitutes a mechanism for isolating, within gas-filled elements, the bubbles which result from the failure of stretched water columns. In view of the short discrete elements characteristic of conifers, the proposed mechanism appears, at first glance, quite reasonable. Bailey (73) has agreed that this mechanism may retard the entrance of gas into the conducting elements, but has shown that in the absence of this action the surface tension of the sap in the pit membranes of conifers may be overcome by pressures of less than 3 atm. Furthermore, in discussing the activity of the torus within the framework of a general theory of moisture movement in large plants, it is of interest to note that this structure is of very restricted occurrence within the plant kingdom, being characteristic only of the Gnetales, Ginkgoales, and certain Coniferales (74). It is of very rare occurrence in the angiosperms. In addition, Wright (75) has shown that in *Ginkgo* the torus may be only one-half the width of the pit aperture and, therefore, that it cannot exert a clos-

XUM

ing action. Curtis & Clark (76) suggest that the chances of ineffective closing of the tori in gymnosperms may be very high, and in addition draw attention to the more far-reaching consequences of bubble formation in the water-conducting elements of hardwoods. They point out that a single break will eliminate an entire vessel, a consideration of some significance when it is recalled that these elements may extend over vertical distances of several feet (76, 77, 78).

Other evidence bearing on the problem of the passage of gases through cell walls has been reviewed by Gibbs (30) and Livingston (79). According to the former, Priestley was unable to displace the liquid contents of closed hardwood vessels by an externally applied pressure of 15 atm. Hungate (21) has suggested from a consideration of cohesion-adhesion-imbibitional relationships in conducting tissues that the resistance to movement of air through cell walls and into vessels might be increased during periods of rapid transpiration. Similarly, Stocking (32) has postulated that surface tension effectively prevents the movement of undissolved gases through hydrated cellulose walls. Alternatively, Woodhouse (80) concluded that gases enter the conducting tissues of transpiring plants prior to the onset of high negative pressures. Lundegardh (54) has suggested that the continuous contact between air and water in plant stems impedes the development of high cohesive forces. Scholander *et al.* (29) have concluded that vessels are pervious to gas during certain periods of the year, but found too that water-filled conducting elements are "shielded from diffusion contact with any evacuated vessels on the long way up the stem."

State of water in conducting elements.—Within the framework of the cohesion hypothesis, in addition to substantiating the existence of a continuous water column or columns under field conditions, it is necessary to demonstrate the incidence of actual tensions in the contents of conducting elements. In this regard Ursprung (81) and Thut (82) have demonstrated a capacity on the part of living branches to raise mercury to heights exceeding 76 cm. under extremely favourable experimental conditions, thus confirming the findings of earlier workers. Similarly Livingston & Lubin (83), Thut (84), and Otis (85) employing artificial surfaces, have obtained similar results. Strong circumstantial evidence of the existence of tensile stresses in actively transpiring material has been obtained by Bakke (86, 87) from observations on the behaviour of plants before and after wilting, and by McDermott (88) from an investigation of the re-distribution of moisture in woody twigs following cutting. Indirect evidence of tensions has been advanced by Renner (89, 90) and Nordenhausen (91, 92, 93) on the basis of experiments with potometers. Values as high as 20 atm. were inferred from a comparison of the rates of flow observed in living samples with those obtained with the aid of a vacuum pump of known efficiency or a resistance yielding a definite pressure drop. Eaton (94) adopting an equivalent approach gained evidence of the existence of tensions of the order of 4 atm. A comparison of exudation rates in de-topped stems under sub-atmospheric pressure and transpiration rates

in entire plants led Warne (95) to predict the development of tensions ranging from 1 to 13 atm. Stocking (32), from a study of changes in the concentration of sucrose solutions injected into hollow spaces in the petioles of squash leaves, reported tensions reaching 9 atm. Crafts (96) investigated the fate of vessel contents subsequent to jarring and deformation of the experimental material, and from the concomitant behaviour of vapor "columns" concluded that water entrapped in conducting elements is under negative pressure. Injection experiments in ash trees led Preston (51) to postulate the existence of tensions of 3 atm. under field conditions. Other evidence pertaining to the existence of tensions in transpiring plants has been reviewed recently by Crafts *et al.* (24).

Circumstantial evidence of the existence of negative pressures in herbaceous plants has been inferred from observed changes in the diameter of vessels with apparent fluctuations in transpirational pull, and in trees from experiments with dendrographs, in which attempts are made to measure the flexure of cell walls resulting from the presumed development of tensions in the water-filled conducting elements [see MacDougal *et al.* (47) for a review of earlier studies].

MacDougal (97) has published extensively on observed diametral changes in the stems and roots of trees with particular reference to Monterey pine. He noted an unusual but characteristic pattern of swelling and contraction in various regions of the same tree over a given time interval, but found the responses (diametral fluctuations) of the differing regions to be markedly out of phase. MacDougal cites as a typical example a root commencing to expand at 8:00 a.m., the lower bole, distant 2.5 m. from the root, at 1:00 p.m., and the upper bole at 7:00 p.m. He also observed a change in this cycle "so that the two phases in the root were at first directly opposed to those of the stem or trunk, then became belated during a month by a displacement of 6 to 8 hours then advanced to the original opposition to the trunk."

MacDougal (97) invited attention to these and to other unusual reactions, and to the difficulties attendant upon an adequate explanation of these phenomena. One of the most curious aspects of his studies is the response of a tree, postulated to be dead, which exhibited "a daily reversible variation which was plainly discernible." However, when placed in a solution of Orange G, very slight upward movement of the stain resulted. MacDougal notes that "if it be conceded that the daily reversible variations in the diameter of a trunk are to be taken as an indication of the existence of a continuous water column under tension in the wood cells, then the above observations constitute evidence of the movement of sap in dead stems, without participation by any living cells whatever." He feels that "while, as Dixon has pointed out, a dead cell or an inanimate mass of colloids does not usually furnish the conditions for maintaining a water column in wood under tension, yet it seems to have done so in this tree," and he contends "that a continuous water column subject to daily variations in tension was present in this tree." In his summary he notes that "the results described in the foregoing paper unanimously

XUM

support the conception of the cohesive column of water of Dixon as the main feature of the hydrostatics and movement of liquids in trees."

Haasis (98) observed a diurnal shrinkage and expansion in the trunks of trees, noting that some parts of the tree were contracting while others were expanding. In a later study (99), employing multiple dendrometers, he attached one instrument in such a manner as to record fluctuations in the diameter of the tree (wood, cambium, and a thin layer of external tissue), a second to trace changes in the woody cylinder, and a third in the inner central cylinder. He observed that diurnal fluctuations in the inner cylinder paralleled those of the tree as a whole, and that the central cylinder appeared to be sensitive to changes in wind velocity but not to very high humidities. Likewise, Haasis noted that despite the fact that the outer wood is commonly assumed to be the main region of conduction, the widest diurnal fluctuations in diameter were characteristic of the central cylinder. His observation that the fluctuations were greatest in the early rather than the later part of the year, at which time the greatest tensions might be expected, may be due to a progressive decrease in the number of water-filled conducting elements over the active season. Haasis reaffirmed that both in Monterey pine and redwood, one region of the trunk may be expanding while another region, distant approximately one meter, may be contracting. In accounting for the unusual behaviour of his experimental material, Haasis appealed to conditions obtaining in the hydrostatic-pneumatic system, stating that the reasons for the divergences in behaviour of different cylinders were not clearly evident.

Subsequently, Karling (100) noted diametral changes in the stems of *Achras zapota*, and observed a rhythmic daily expansion and contraction of the trunk under more or less constant weather conditions. He also found that precipitation, higher humidities, temperature changes, and variations in wind velocity altered greatly the rhythm of fluctuations in trunk diameter, and he correlated both daily reversible and seasonal variations in diameter with weather conditions. Similarly, Friesner (101), as a consequence of dendrographic studies, has attributed hourly variations in the stem diameter of trees to turgidity changes in cells resulting from high transpiration losses. Recently, Fritts & Fritts (102) have investigated fluctuations in the stem diameter of beech trees over the growing season. They noted a progressive increase in the amplitude of the diurnal variations in diameter with decreasing amounts of soil moisture and improved conditions for evaporation. These authors have concluded that daily reversible fluctuations in diameter are directly correlated with internal water relations and turgidity changes within the tree. On the microscopic level, Bode (17) observed changes in the diameter of vessels with fluctuations in transpirational pull.

Priestley (65) has reviewed in some detail the evidence which has been presented in support of the existence in transpiring trees of continuous columns of water under negative pressure. He contends that neither the experiments of Boehm, Ursprung, and others, dealing with the lifting of mercury

columns by living shoots to distances exceeding barometric height, nor the researches of Bode and Holle regarding the contents of conducting elements are indicative of the existence in transpiring trees of large negative pressures in continuous water columns which occupy vessels and tracheids. Alternatively, he has concluded, on the basis of experimental evidence, "that the tracheae contain gas at low pressure rather than liquid columns under high tensions."

Similarly, Scholander *et al.* (29) have recently undertaken an extended analysis of observations purportedly confirming the incidence of tensile stresses in the contents of conducting elements in actively transpiring plants. Their comments with respect to the potometer experiments of Renner, Bode, Nordenhausen, and others are of particular interest, and they have offered several pertinent criticisms with regard both to methodology and interpretation. Employing the technique of Renner (89), Scholander *et al.* have shown that apparent negative pressures of considerable magnitude (2 to 20 atm.) may be demonstrated, not only in leafy twigs given a prolonged air embolus, but also in leafless samples with gas-filled vessels, and in sections open at both ends with air-filled vessels. The apparent existence of tensions in such material led Scholander and associates to comment upon the design of experiments of this nature, and to criticize attempts to interpret the results thus obtained in terms of the state of water in conducting elements.

The results of recent experimental studies are of interest in the light of the proposed functioning of the cohesion mechanism: see also Pfeffer (5) for a brief review of earlier experiments of a similar nature. Elazari-Volcani (103) severely interrupted large percentages of the total conducting area of citrus fruit trees by means of unilateral and circular incisions into the stem. Severance of as much as 70 per cent of the conducting tissue resulted in very slight wilting, leading to increased conduction in the central region of the stem and pronounced lateral transfer of moisture. Preston (52) repeated these experiments utilizing fairly large trees, and supposedly ensured complete interruption of all water-conducting elements (vessels) by means of overlapping saw cuts made from opposite sides of the bole. He confirmed the findings of Elazari-Volcani (103), and concluded from his experiments that either liquid columns are not necessary for moisture movement and that the ascent of sap is due to some mechanism other than that envisaged by Dixon (9, 10), or that new columns may be developed quickly enough to prevent exhaustion of stored water above the saw cuts. Preston concluded that the latter alternative was the more feasible, and has included a diagram showing the proposed orientation of conducting elements laid down subsequent to the double saw cut procedure. In discussing his results, Preston has emphasized the great importance of an actively functioning cambium and the development of new conducting tissues to the continued moisture movement in trees which have been treated in the above manner.

Greenidge (104, 105) carried out a programme of studies similar in design to those of Elazari-Volcani (103) and Preston (52), but investigated the re-

XUM

sults of experimental treatment by means of the injection of water-soluble dyes into the lower boles of treated trees. Utilizing forest grown trees of considerable size, Greenidge interrupted all vessels in the stem by means of two or four over-lapping saw cuts arranged in varying patterns along the bole. In trees receiving two saw cuts, the incisions were distributed in such a manner that the distance between the upper saw cut and the injection level was less than the length of the unit vessel in the species under investigation, and thus the vessel contents were presumably exposed to atmospheric pressure. Trees were injected from 20 min. to 12 days after saw-cutting. Despite these drastic treatments the dye solution moved quickly and without difficulty into the apices of all trees injected. A comparison of the staining patterns in treated and untreated trees revealed that repeated mechanical interruption of vessels exercised unexpectedly little influence on conduction, resulting simply in increased lateral transfer of moisture and a reduction in the rates of moisture uptake. Rates of vertical travel of moisture were decreased slightly by surgical treatment of conducting tissues (106).

In other experiments Greenidge (105) observed continued moisture movement in the stubs of treated and untreated trees, the upper boles and tops of which had been removed prior to injection.

Scholander *et al.* (29) investigated the effects on water conduction of severe interruption, by gas emboli, of the normal channels of transport. Large vine stems were severed under water, stepped in a supply of liquid, the rates of uptake measured, and the plants then left to absorb air or helium for varying periods. Despite the resulting presence of large volumes of gas in the stems, rates of uptake diminished slightly or not at all when the vines were replaced in a supply of water. Other plants were severed from their roots, left for a short period, placed in water, and rates of uptake observed. "Next day" the plants were removed from water and allowed to transpire in air until wilting occurred. Upon being returned to water, uptake continued at a much reduced rate but turgidity of leaves was regained within a period of 20 min. to 1 hr. Continued, much reduced rates of uptake over the succeeding four days suggested to Scholander *et al.* (29) that much of the sap flow was confined to the "finer structures between the vessels."

In other experiments these investigators allowed a large severed vine stem to transpire in air until wilting occurred, then placed the plant in a 10 per cent solution of copper sulphate. The poison moved to the top of the vine discolouring the leaves. These workers point out that "neither poison nor a vascular stream broken by large amounts of air could prevent the vine from getting fluid above atmospheric height and into the vessels and leaves." In a second plant the rates of uptake of water and copper sulphate were of the same order suggesting "there were no metabolic sap pumps at work in the stems."

Some additional comments on the cohesion mechanism.—Notwithstanding the favourable reception afforded the cohesion mechanism at the time and subsequent to its presentation, several workers have found various aspects

of the concept untenable, and, in certain instances, have advanced telling arguments in support of their views. A number of criticisms of specific points of the concept have been listed above. In addition, Woodhouse (80) has found the mechanism lacking in certain essential features, and has indicated the need for additional postulates. Priestley (65) has rejected entirely certain of the basic assumptions of the liquid tension concept such as the existence of continuous columns of water, and has concluded that the cohesion hypothesis does not rest upon a satisfactory experimental foundation. Similarly, Preston (51), as a consequence of empirical considerations, has suggested the need for a review of the entire problem of the ascent of sap. In a second paper, Preston (52) has reviewed in considerable detail the several factors which have prompted him to recommend the complete rejection of the classical cohesion mechanism. Lundegardh (54) has concluded that only fragments of the original cohesion concept can be retained in a satisfactory mechanism in explanation of the ascent of sap. In a very recent paper Scholander and associates (29) have discussed several shortcomings of the cohesion mechanism, and have marshalled evidence at variance with each of the fundamental requirements of the concept. In their view, direct evidence in support of the theory is lacking, and they contend that in the case of large plants the problem of the ascent of sap remains unsolved. Admittedly, much of the evidence thus far advanced in support of the concept is of an indirect nature, gained largely from plants of low stature. In this regard, problems stemming from the nature of the actively transpiring plant as a physical system render it difficult to obtain reliable information regarding the actual behaviour and requirements of large woody stems under field conditions, and to gain unequivocal experimental evidence to support or refute a particular aspect of the mechanism. Thus in arborescent forms in particular, the potentialities of the concept remain to be clarified.

Recent Alternative Explanations of Sap Ascent

The potentialities of several earlier "vital" mechanisms advanced in explanation of the ascent of sap have been discussed by Dixon (9, 10) and others (5, 8, 30, 31). In addition, Ewart (64) has presented an extended review of the problem. Finding the cohesion mechanism lacking in several respects, he speculated upon the importance of the role played by living cells in the transport of water in trees. Although convinced of the necessity of a pumping action to drive moisture to the tops of large plants, Ewart was unable to demonstrate such a mechanism, and he felt that the character and diffuseness of the mechanism rendered its existence difficult to detect. Similarly, Ewart was unable to confirm Strasburger's (6) results with regard to the movement of poisons in supposedly dead trees, evidence which has been utilized with significant effect by supporters of physical concepts in explanation of the ascent of sap, and he concluded that the upward movement of water is a "vital" problem insofar as it depends upon conditions which can only be maintained in living wood.

XUM

The importance of living cells in the process of water conduction in plants has also been stressed in a theory advanced by Bose (107). He reported a rhythmic contraction and expansion in the cortical tissue of *Desmodium gyrans*, the amplitude of these fluctuations being amenable to control by variations in pressure and temperature, and in some measure to control by anesthetics. Bose also observed that the upward movement of moisture appeared to be directly linked to these fluctuations, conduction being accelerated or retarded by the same factors which governed the fluctuations in rhythmic activity. In consequence Bose concluded that the ascent of sap was a vital as opposed to a purely physical process. Again, observing that the cortex appeared to be the seat of pulsatory activity, he suggested that this tissue was the locus of moisture movement in plants rather than the xylem which did not appear to suffer expansion and contraction.

Molisch (108) reported confirmation of certain of Bose's results, observing that moisture movement was closely linked to a characteristic rhythmic activity of the plant which was amenable to control by drugs. Areas thus stimulated were considered to act somewhat like pumps driving water toward unstimulated regions. Molisch like Bose concluded that the mechanism of the ascent of sap was physiological rather than physical in nature.

The concepts of Bose (107) and Molisch (108) have been destructively criticized by a number of investigators including Smith *et al.* (20), Benedict (109), MacDougal *et al.* (47), and others. Smith *et al.* (20) have suggested that Bose's interpretation of certain electrical phenomena, which the latter observed, may be in error, and they point out that Bose's contention that sap ascent occurs in the cortex is at variance with the experience of others concerned with this problem. MacDougal *et al.* (47) and Miller (31) have criticized Bose's work from the standpoint of both methodology and interpretation. Utilizing a wide range of material (10 species), Benedict (109) found that the actual rates of moisture movement in trees are several thousand fold more rapid than those which are to be anticipated as a consequence of Bose's hypothesis.

There appears to be no currently acceptable hypothesis of sap ascent in large plants which hinges on root pressure as the motivating force in moisture movement. Within recent years such considerations as the absence of the phenomenon in actively transpiring plants, the restriction of evidence of the process to the lower reaches of the stem, and the fact that root pressure has seldom, if ever, been observed in gymnosperms (110) have led Kramer (27) and others to limit the importance of the mechanism (11, 76, 111). The role of root pressure in the re-filling of the stems of large woody plants during the dormant period is likewise still to be clarified (10, 30).

Dissatisfaction with several basic considerations of the cohesion concept led Priestley (65) to formulate and describe very briefly certain features of an alternative mechanism of moisture movement. In Priestley's view the upward movement of moisture is closely linked to the development of buds and foliar surfaces, conduction taking place via the maturing elements laid

down in a specific year. He suggested that the differentiating vessels exhaust the water contained in the older wood, and that the newly-formed elements force this moisture, in some manner thus far unexplained, into the developing leaves. The results of injection procedures led Priestley to conclude that the conducting elements of the older wood act as reservoirs, becoming gas-filled (chiefly water vapour at sub-atmospheric pressures) once relieved of water, and playing no role in the process of water conduction. With the degeneration of their protoplasts, newly-formed tracheae may also be injected, according to Priestley, a result which, in his view, suggests that their liquid contents have either moved into transpiring leaves or into more newly-formed elements developing centrifugally. The dependence of the mechanism on growing and developing tissues is repeatedly emphasized by Priestley.

Priestley's hypothesis is largely speculative in nature, and the observations presented in support of his conclusions are in need of extended consideration. Field observations regarding the continued movement of moisture in trees following surgical treatment of conducting tissues, the lateral transfer of moisture, and the pronounced movement of moisture in the inner rings of certain species (104, 112) are not consistent with his views. Likewise, it is doubtful that the fortuitous coincidence in time between the cessation of cambial activity and foliar development, and the termination of upward moisture movement demanded by Priestley's mechanism obtains in the field.

Lund (113) investigated the possibility that a continuous electrical current directed upward in the wood of plants supplies the energy for an electro-endosmotic flow of sap in an upward direction in the conducting elements, and to a downward flow in the cortex as well as a lateral transport across the stem. A careful analysis of electro-kinetic considerations revealed that all physical factors involved would support the concept. However, his data, while of a preliminary nature, are consistent with other interpretations of sap ascent, and the rates of travel he obtained appear to be much too small.

Peirce (50, 114) has discussed at some length the complexities of the problem of the ascent of sap in plants, and has stressed the viewpoint that the phenomenon is not purely physical in nature. His conception, however, cannot be termed vitalistic. He has concluded that while water is moved either in the liquid or vapour phase through the plant by physical means, when the living cells surrounding the vascular tissues are killed by heat, cold, or poisons, the conducting system is rendered functionless and the plant dies. In Peirce's view water moves through the plant but is not pumped or pushed; the living cells maintain a continuous but many phased water mass in the capillary body of the plant, conditioning but not compelling the ascent of sap.

Handley (115), as a consequence of experiments with chilled ash and maple saplings, has advanced the view that living cells are required for the upward movement of moisture in plants. With the use of a suitable refrigeration technique Handley was enabled to lower the temperature of the stems of his test plants to $-2°C.$, and he noted that decreasing the temperature be-

XUM

low 2°C. resulted in a drastic interruption in the supply of moisture to the leaves, and to marked wilting. Raising the temperature of the saplings above 2°C. led to a complete recovery of the wilted foliage, indicating that the cause of wilting was not death of the leaves. To Handley a temperature of 2°C. appeared to mark a significant level with regard to the mechanism of moisture movement. He concluded that it was unlikely that such a small temperature range could alter materially "the physical condition of continuous water columns in the vessels, if indeed such are present and in any way connected with the ascent of sap." He felt that because he had not employed sufficiently low temperatures to freeze the water in the vessels, then moisture movement to the leaves from the continuous water columns in the vessels should have continued. Wilting, however, occurred. Handley could not attribute his results to marked changes, over a two degree interval, in the viscosity of water, a view subsequently adopted by Preston (52), and he observed also that his results were not consistent with Peirce's (50, 114) contention that moisture moves in trees in the vapour phase, or with Priestley's (65) concept of the mechanism of moisture movement. He concludes that living cells are involved in the ascent of sap, and that the process "involves a chain of living cells continuous from roots to leaves." Likewise, Handley suggested that vessels and supporting elements are not active in water conduction but function only as reservoirs, a viewpoint somewhat similar to that expressed by Priestley (65).

Preston (52) has considered at length the significance of Handley's findings, particularly as they bear on mechanisms of moisture movement which turn on considerations of passive flow under external forces. In Preston's view, "no property of water which can affect its flow changes sufficiently rapidly at these temperatures to cause water to become deficient in the leaves." The reverse view, however, appears to have been held by Pfeffer (5). Handley's observations together with others discussed by Preston (52) have prompted the latter to conclude that only living xylem is involved in water transport, and to call for the complete discarding of the "classical Cohesion Theory." It is questionable, however, whether Preston's analysis is sufficiently precise to justify this view.

Recently, Lundegardh (54) has presented the results of an extended study of moisture movement in trees. In his view the vessels and larger tracheids of large woody dicots are air filled and play no role in conduction, movement being confined to the narrower tracheids which contain water and form a continuous water path from roots to leaves. He concludes that a very small proportion of the total water in a stem is mobile and that the transpiration stream is confined to this relatively free fraction. Rejecting the cohesion hypothesis, Lundegardh makes appeal to the complex of capillary suction in the medium sized tracheids, electrocapillarity and other surface phenomena, and the activity of living cells to explain the movement of water in trees. In his view, high suction forces originating in the leaves are not required for moisture movement, and he contends that "capillary forces in co-operation

with the adsorptive qualities of the wall substance and the osmotic imbibition of the living tissues in the stem are so successfully contributing to the maintenance of a continuous sheath of water that an ascending sap stream can be maintained by a real suction pressure, originated in the transpiring leaves, of moderate height, perhaps even less than one atmosphere."

A critical appraisal of the numerous points raised by Lundegardh (54) is not possible at this time, and must await the extended efforts of both anatomists and physiologists. However, certain of his suggestions may be questioned on the basis of present knowledge. Although the supporting elements of large woody dicots may function in conduction under certain circumstances (5, 8, 24, 29), Lundegardh's contention that vessels play a minor, if any, role in moisture movement would appear to be contrary to the experience of several investigators. Strong circumstantial evidence of the importance of vessels in conduction has been advanced recently by Banfield (116), Beckman *et al.* (71), Struckmeyer *et al.* (117), and Bailey (118). Finally, Lundegardh's suggestion that the medium sized tracheids and not the vessels form the main channels of transport in trees, is not in keeping with the marked differences observed in the rates of moisture movement in dicots as opposed to those in conifers (70).

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DROUGHT RESISTANCE IN PLANTS AND PHYSIOLOGICAL PROCESSES[1]

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Introduction

The term "drought resistance" is variously defined and there are also different methods employed for its determination. Some (2, 14, 26) deprive plants of water in the laboratory or place them in chambers with variable relative humidity and determine the loss of water to a point from which the plants are unable to recover; others seek the permanent wilting point i.e., the moisture content of the soil at which the plant is unable to recover its turgor. Such methods do not measure drought resistance but resistance to desiccation. Moreover the recovery from wilting does not indicate the re-establishment of all normal functions, as will be seen later.

Others define as resistant species those which develop normally in dry habitats and yield maximum crops (65, 94). In such cases the manner in which best results are achieved is not important. Plants adjust themselves to drought in various ways such as by limiting transpiration to a minimum, or by getting sufficient water from the deep soil layers (18, 65). Neither a morphologic nor physiologic adjustment is necessarily a property of all xerophytes (11, 62, 65, 80). A study of the adjustments of plants to drought can be of great importance yet only a small number of them possess such abilities.

In the present report consideration will be given to the influence of drought upon certain physiologic processes and upon such reactions as stomatal function, photosynthesis, respiration, the metabolism of carbohydrates, osmotic effects, and desiccation of tissues.

Variations in the Water Content of Plants

The water content of a plant is subject to diurnal variations during its entire growth period (7, 32, 51, 57). In nature the water deficit may reach 15 or 20 per cent, sometimes 25 or 30 per cent or even 40 per cent (total fresh weight basis). In warm dry air transpiration is increased and absorption of water from the soil does not compensate for that which is lost. Then wilting occurs, and it may be temporary or permanent. Yet in a moist climate, as in northern Russia, the deficit at midday may reach 28 per cent (52).

In plants well supplied with water the minimum water content is reached between 2:00 and 4:00 P.M. and the maximum between 2:00 and 6:00 A.M. (104).

[1] The survey of literature pertaining to this review was concluded in August, 1956.

Stomatal Movement

Stomata exercise a role of great importance in absorption of CO_2 from the air and in evaporation of water. Their movement is regulated by light and the amount of water in the plant. When the leaves are saturated with water the stomatal aperture is proportional to the light intensity (88). But intense light can induce in certain plants, as in coffee, closure of the stomata (73). In general, a water loss of 10 per cent (fresh weight basis) by a plant induces closure of stomata (75, 76); in certain species, as in *Vicia* and *Chrysanthemum*, the stomata close when the loss is as small as 3 to 5 per cent. Variations in different species can be very marked, depending upon the species, its stage of development, and the physiologic processes within the stomatal apparatus. Shade leaves upon a given tree are more sensitive than those growing in the light.

The movement of the stomata depends upon the structure of the guard cells and their changes in turgor brought about by enzyme activity (29). Starch accumulates in guard cells of closed stomata, which is changed to sugar by enzymes when stimulated by light and by degree of turgescence. As a result the concentration of cell sap in increased, the volume of the guard cells is increased, and the stoma opens. Darkness and lack of water stimulate the reverse processes; the sugars are then transformed to starch, pressure is decreased, and the stomata close.

The osmotic pressure and the content of starch varies between species and even in the same species. The sap concentrations, expressed as moles of KNO_3, in stomata having a maximum opening may reach 3.0 *N*, although that of the epidermis and mesophyll may be only 0.4 to 0.6 *N*. In a dry atmosphere, however, or in darkness or near the end of the day, the guard cells may have an osmotic pressure like that of the epidermis and mesophyll. These observations on the physiology of stomata have been confirmed by various investigators (55, 56, 89, 95).

A lapse of 1.5 to 2 hr. occurs while the enzymatic processes are very slowly operative in the movement of stomata (1, 29). But there is indication that the enzymatic process can be hastened (103). Plants with widely-open stomata, transferred from a humid atmosphere to a dry one, lose water very rapidly and wilt within 3 to 5 min., while the stomata remain completely open. Before closure of the stomata, under these conditions, the water loss may be as much as 30 to 40 per cent.

The correlation between the quantity of starch in the guard cells and the size of the stomatal opening is not always perfect (103). But the decomposition of starch is accomplished by stages and its disappearance does not signify that it has been completely converted to sugars or that the maximum osmotic value has been reached. Dextrines are first formed, which are converted to disaccharides and finally to monosaccharides.

Phosphorylating enzymes, in particular, are very active in guard cells (108).

Very important factors in regulation of stomatal activity are the cations

and anions of mineral salts (31a). The passage of solution of electrolytes into tissues stimulates the transformation of starch to sugars, increases the osmotic pressure, and amplifies the stomatal openings. Both hypertonic and hypotonic solutions exert an influence. Thus, when sections of epidermis of different species, having the guard cells filled with starch, are placed in 0.3 to 0.4 *N* KCl they show plasmolysis primarily; but the starch disappears gradually, the osmotic value increases to 1.3 *N* and the stomata open widely within 2 or 3 hr. In a hypotonic solution of 0.05 *N* KNO_3, the starch also disappears; the stomatal openings increase but the process requires 36 hr. Solutions of 0.15 to 0.30 *N* have the stronger effect, and in weaker or stronger solutions the process is retarded. The passage of water causes the starch to accumulate and brings about closure of the openings as a result of the leaching out of the salts.

The salts of potassium with different anions, taken in isotonic concentrations, give the same effect. Solutions of various sugars do not induce the opening of stomata; in the presence of strong concentrations the starch is broken down but the stomata do not open.

Li, Na, Cs, and Rb, of Group I (Periodic table) act as does potassium, that is, they favor the hydrolysis of starch to sugars and cause a large increase in osmotic value. Li is the most active, after which in order come Na, Cs, K, and Rb. The action of metals of Group II is very different. Beryllium functions like the elements of Group I, but in very weak concentrations; a solution of 0.0001 *M* slowly stimulates the hydrolysis of starch and the opening of stomata, but only very gradually during a period of 22 hr. A concentration of 0.001 or 0.002 *M* acts more rapidly, whereas at a concentration of 0.05 *M* the process is completed within an hour. Magnesium influences opening only slightly, and calcium and strontium are without effect. Barium in dilute solution can stimulate the digestion of starch and stomatal opening. Each of these four elements in high concentrations induces the disappearance of starch but without causing the stomata to open, acting like solutions of sugar, or like the loss of water.

Members of Group II, Mg, Ca, and Sr, are antagonists of Group I; in weak concentrations they nullify their activity. By the use of salts, changing their concentration and the relation among the elements, one can control stomatal movement.

Influence of Drought on the Functioning of Stomata

A plant exposed to severe drought is not able to re-establish its normal functions, but remains abnormal even though it regains its turgescence (31). The stomata open slightly or partially lose this function and may die; thus in *Centaurea orientalis* 8 per cent retained ability to open, 73 per cent were closed and 19 per cent were killed. In certain other species up to 45 per cent of the stomata were rendered inactive although the leaves appeared to be perfectly normal. These observations were repeatedly confirmed, using the porometer of Darwin & Pertz (13).

XUM

As previously indicated, the loss of water by the plant stimulates the transformation of sugars to starch, and lowers the osmotic pressure. But when the wilting passes certain limits, the starch in the guard cells, as in the mesophyll tissues, decomposes. Species differ among themselves in their reaction to drought. Decomposition is complete in species adapted to moist habitats when the water loss ranges from 15 to 30 per cent (fresh weight basis). In species more resistant to drought this phenomenon may occur within the range of 50 to 60 per cent water loss. Moreover there is a marked difference between these two types of plants in the rapidity of starch digestion, the process being more rapid in the former group (32).

The immersion of leaves in water stimulates, generally, the accumulation of starch in widely-opened stomata. Previous wilting retards the process. In an experiment, wilted leaves were placed in stoppered flasks to check further wilting. After different elapsed times they were placed in water. Leaves which were not wilted formed a fixed quantity of starch in 4 hr.; those wilted for an hr. required 20 hr. to form an equal amount of starch; those wilted for 4 hr. required 44 hr.; and those wilted for 20 or more hr. failed completely to form any starch (32).

In other experiments (38) sections of epidermal tissue were placed in microchambers having different relative humidities. The starch digested gradually at a relative humidity between 99 and 94 per cent, the osmotic pressure increased as did the stomatal apertures but there was no change at 100 per cent relative humidity. The osmotic value at 100 per cent relative humidity was equivalent to 0.12 to 0.15 *M* $CaCl_2$, whereas at lower relative humidity it was equivalent to 0.3 to 0.4 *M*, the range extending to 2.0 *M*. Drying stimulates the progressive decomposition of starch. At first polysaccharides of high molecular weight are formed, followed by the formation of di- and monosaccharides.

The stimulus of drying, as affecting breakdown of polysaccharides, is not of short duration but persists for a time after the tissues are in contact with water. If the epidermis was placed for a brief period in a relative humidity of 92 per cent and was then placed in a relative humidity of 100 per cent, the starch gradually disappeared, and the osmotic pressure increased as did the aperture of the stomata, none of these changes occurred in tissues continuously kept at 100 per cent relative humidity (31).

In other experiments the epidermis was placed in different concentrations of saccharose ranging from 0.2 to 2.0 *M* for 2 hr. and then transferred to water. The stomata were closed and there was little decomposition of starch when the solutions ranged from 0.2 to 0.3 *M*, and were widely open, with marked decomposition of starch when the solutions were within the range 0.75 to 1.5 *M*.

Similar results were obtained with intact leaves placed in stoppered flasks to control relative humidity for varying periods, and then immersed in water. When the wilting was slight and had just began, starch was not broken down, the stomatal apertures were unchanged, and osmotic pressure

was not increased, but when drying was marked and continued over a long period, the stimulus for inciting these processes was provided.

Carbohydrate Metabolism in Green Tissues

Many investigators have observed that starch disappears from wilted leaves (68, 69, 71), following decrease in soil moisture (59, 83, 106) or the addition of a salt solution (NaCl) to the soil. Sugars accumulate simultaneously (17, 36, 47, 59). After water is furnished, as by irrigation, if the water loss is not too severe starch formation will become re-established (99). Drying stimulates the activity of amylase (85) and phosphatase (84), not only in the guard cells but throughout the leaf.

A comparison of mesophytes and xerophytes shows that there is a difference between these two groups of plants (36). The former are more susceptible to wilting, and the decomposition of starch requires a lesser loss of water and a shorter period of stimulation. Xerophytes are more tolerant to water loss, react less actively, and require a greater stimulus.

The loss of water intensifies decomposition, inhibiting assimilation. Thus in an experiment, plants were placed in darkness to permit them to lose their starch. Then the leaves were removed, and cut in half, one part being kept turgid in stoppered flasks and the other half being permitted to wilt to a certain degree. Turgescence was re-established rapidly when these leaves were placed in light, in water, or on the surface of a 1 per cent glucose solution. Under these conditions starch accumulated rapidly in each, but synthetic activity was inhibited to a degree in the half leaves that had been wilted. Starch formed more slowly and in lesser amount in the wilted leaves, and when the wilting was severe there was no recovery of synthetic ability. Mesophytic species were more sensitive to water loss than were xerophytes. When the water loss was only 11 per cent, synthesis in *Hieracium* was reduced.

In another experiment, leaves deprived of starch by darkness were placed on the surface of different concentrations of saccharose to which 0.06 *M* glucose had been added to facilitate the synthesis of starch. Leaves of 40 species of plants from different localities were used in this experiment. Synthetic activity was reduced by the concentrated solution but was not completely eliminated. Concentrations of 0.3 to 0.4 or 0.5 to 0.6 *M* inhibited starch synthesis in hydrophytes and mesophytes, respectively, whereas concentrations of 1.1 to 1.4 *M* were required for xerophytes.

The accumulation of carbohydrates in plants depends upon several factors. An increased supply of available nitrogen stimulates the utilization of carbohydrates, and if, in addition, sufficient moisture is available, growth and the formation of new organs are accelerated. On the other hand, if sufficient moisture is not available, growth is interrupted and polysaccharides tend to be accumulated (10, 105). The breakdown of carbohydrates in the leaves may be accompanied by its deposition in the roots (17, 23, 47). It is not possible to state that water loss induces the breakdown and disappearance of

XUM

starch in all parts of the plant, but it can be said to take place in the leaves of the majority of species. This question needs further study.

Determinations reveal that plants growing in dry habitats contain more sugar than those in moist habitats. Studies (35) made in Czechoslovakia and France of 122 species of plants yield the results as presented in Table I. Marked variations occur within each group but these differences disappear when averaged. These variations show that it is not possible to say that all xerophytes are able to produce a high content of sugars. One can only say

TABLE I

HABITAT AND SUGAR CONTENT

Plant Groups	Number of Species	Range in Sugar Content*	Average Sugar Content*
Succulents	8	0.46– 0.98	0.72
Herbaceous Mesophytes	22	0.47– 2.55	1.25
Herbaceous Xerophytes	26	1.23– 3.73	2.64
Mesophytic trees and shrubs	38	1.48– 8.21	3.60
Xerophytic trees and shrubs	27	3.80–11.56	6.89

* Per cent of sugar content based on dry weight.

that the majority of such species have this ability. The sugar content of succulents is low. Trees and shrubs have almost three times as much sugar as do herbaceous species.

A single species is able to modify its sugar content significantly depending upon the humidity of its habitat. We find significant differences when a species is grown in a moist, a moderately dry, or a dry situation. Under these three conditions *Rumex acetosa* has a sugar content of 0.69 per cent, 1.04 per cent, and 3.53 per cent respectively; *Ficus carica*, 1.62 per cent, 4.35 per cent, and 7.05 per cent, respectively; and *Hedera helix* 2.93 per cent, 3.92 per cent, and 7.7 per cent, respectively.

By reducing the water in plants under controlled conditions one can increase their sugar content. Thus in three species of Rumex, after losing 20 per cent of their water, there was an increase in sugar concentrations of 49 to 96 per cent, after several hours. When *Inula viscosa* was placed for 36 hr. in a range of different moisture conditions the leaves showed sugar concentrations of 0.015, 0.021, 0.042, and 0.053 *M*. The leaves in humid atmosphere contained only disaccharides and those in a dry atmosphere had both di- and monosaccharides (35).

PHOTOSYNTHETIC ACTIVITY

Drought influences photosynthesis, first, by decreasing the size of the stomatal openings which limits CO_2 absorption, and second by lessening the photosynthetic activity of green tissues.

A comparison of photosynthetic rate of fresh plants with wilted ones having closed stomata (30, 32, 44) shows that marked differences exist between them. A loss of water of 16 to 47 per cent or more causes a decrease of 20 per cent in rate but there does not seem to be a close correlation between the amount of water lost and the intensity of photosynthesis. Plants which have recovered from wilting do not carry on assimilation normally but their capability to do so is reduced by 59 to 35 per cent.

Two-year-old apple trees in pots in soil having a favorable moisture content (36.4 per cent) were deprived of water (83) and the soil moisture content declined slowly to 15.2 per cent. The stomata closed and the photosynthetic rate decreased first by 19 per cent, then by 50 per cent, and at time of wilting by 85 per cent. When water was applied turgor was restored, and photosynthesis was restored to 75 per cent of normal. After a week the apple trees had completely recovered.

In similar experiments with pecan (58) reduction of soil moisture lowered the photosynthesis rate but the trees did not return to normal after water was applied. Similar reactions occurred with potato (9). If potatoes remained wilted for 24 hr. and were then watered they recovered completely in 4 hr. If they remained wilted for 72 hr. then 48 hr. were required for recovery. Water loss caused reduction in photosynthesis also in buckwheat (97), in pine (50) and in tomatoes and beans (106). In corn, the rate was reduced to 37 per cent of normal (98).

Photosynthesis is reduced also in plants without stomata, such as mosses and lichens (86). Three species of the marine alga, *Fucus*, were air-dried for 15 min. after which they remained in water for 5 hr. (69). One of them was then found to have recovered 97 per cent of its normal photosynthesis, another 72 per cent, and the other 42 per cent. Longer drying than 15 min. caused a greater reduction in photosynthesis. Other species of marine algae are also susceptible to reduction in photosynthesis after loss of water.

Stomatal movement is a very important factor in photosynthesis. There is a certain relationship between size of the aperture, photosynthesis, and transpiration. This relationship changes in different habitats. In their normal habitat, with humid air, mesophytes transpire less than xerophytes in their normal habitats (32, 43, 44). Thus the former lose 7 to 11 gm. of water in assimilating 1 ml. of CO_2, and the latter 19 to 84 gm. Values for mesophytes growing in dry sites are much higher than those of xerophytes. *Aristolochia clematitis* in a moist site evaporated 11 gm. of water per 1 ml. of CO_2 and in a dry site 351 gm. One other species evaporated 30 and 130 gm. in these two sites and another 41 and 415 gm. of water respectively, although three species of xerophytes in a dry habitat evaporated only 35, 56, and 64 gm. of water, respectively.

If the sites are too dry, the photosynthesis of xerophytes is decreased. Thus, *Stipa pennata* absorbed 59 ml. of CO_2 in a normal habitat, and only 17 ml. in an extremely dry one. Two other grasses under the same conditions yielded values of 103 and 48 gm., and 138 and 90 gm., respectively.

XUM

Other investigators have obtained similar results (91). Xerophytic species produce a large weight of organic substances per unit of water transpired (96) since the large number of stomata favor the entrance of more CO_2. Comparison of mesophytic and xerophytic grasses (60) showed the latter have the greater photosynthesis. Various investigators (18) state that xerophytes, plants having better development of palisade and of chloroplasts, are better able to absorb CO_2.

A constant relationship between photosynthesis and transpiration has been sought on the assumption that the two processes are more or less equally dependent upon permeability of the epidermis to gases. But the reaction of these two processes to environmental conditions is not alike. For example, when the air is saturated with water vapor, transpiration is checked but there is no modificatory effect upon the absorption of CO_2. The size of the apertures, the intensity of the light, and its composition, the temperature, and the water content of the leaves influence these processes differently.

Transpiration and photosynthesis of young apple trees (25) are decreased by drought, but the former is decreased to 50 per cent and the second to 25 per cent of normal. The transpiration of *H. helix* and of *Impatiens pariflora* is decreased to 5 to 15 per cent, but with no change in photosynthesis (8). In experiments with potato and cabbage (93), favorable soil and air moisture stimulated the opening of stomata, evaporation, and photosynthesis but under conditions of drought, photosynthesis fell to a very low value although transpiration was increased.

The leaves of *Pelargonium* (79) were exposed to light, after the plants had first been kept in darkness and hence did not contain the products of photosynthesis; the stomata opened gradually, and the photosynthetic rate increased, the curves paralleling each other. There was also a good correlation between stomatal opening and photosynthesis in leaves of *Prunus lauro-cerasus* (61).

Carbon dioxide enters the leaves not only by way of the stomata but apparently also through the epidermis when the stomata are closed (67). A careful comparison of different species (20) having stomata either on one or both surfaces shows that assimilation is more active through the surfaces with open stomata than through those without stomata. Photosynthesis is checked completely by a well developed cuticle. Leaves with a delicate cuticle are able to assimilate equally well whether the stomata are open or closed (15, 25, 83).

Respiration

As previously indicated, the loss of a large amount of water by a plant induces the breakdown of polysaccharides to simpler compounds of another kind than sugars. Starch may disappear without accumulation of sugars. The probable products are on the way toward becoming the final products of respiration, namely water and carbon dioxide.

Older investigations, based principally upon studies on seeds and seedlings, show that a decrease in respiration accompanies a reduction of water content of tissues. But results are otherwise when such organs as green leaves and stems are used. Species of hydrophytes or mesophytes show increased respiration when wilted (33), a result not found for xerophytes. Thus this question needs further study.

The following data show that *Ranunculus repens* responds very markedly.

Loss of water, per cent of water content	7	22	28	29	34	36
Increase in respiration, per cent of control plant	7	27	45	65	66	74

Other species react similarly, respiration increasing 26 to 30 per cent to as much as 40 to 50 per cent. Xerophytes were observed to react very differently, there being little or no change in respiration, which could be either less or more, following a water loss of 20 to 52 per cent.

A comparison of absolute values shows that xerophytes use a lesser quantity of organic substances in respiration than do mesophytes. Thus, calculating the quantity of carbohydrates of the formula $(C_6H_{10}O_5)n$ consumed in respiration, xerophytes may lose 4.0 to 9.0 per cent (an average of 5.7 per cent) of their dry weight in 24 hr., whereas mesophytes lose 7.7 per cent to 15.4 per cent, an average of 10.5 per cent. These calculations are based on more than 200 determinations. One must not insist that all xerophytes have a lower respiration rate than mesophytes, but only that it occurs commonly.

The daily loss of organic material through respiration can be as much as 2 to 10 per cent of the dry weight (33). The majority of our determinations have been made when the temperatures were moderate, but some were made when temperatures were high. Respiration is a very important factor in crop production because if the dry weight of vegetation produced on a hectare is 10 tons then the daily loss can be 500 or more kg.

Dry weather creates very unfavorable conditions for plants. The stomata may remain closed throughout the entire day, consequently photosynthesis can proceed at only a low rate. The lack of water can provoke a high rate of water loss and wilting which decreases assimilatory activity and stimulates the breakdown of organic substances through respiration. High temperatures throughout the day and night also stimulate the consumption of organic substances. As a consequence of such unfavorable conditions there will not be any increase in dry weight, and it may even be decreased. Daily determinations of dry weight of field grown wheat (1000 samples per day) show that there is a decrease in dry weight of the entire plant during very warm dry weather, and that the weight of the crop is reduced. Respiratory intensity is at a maximum at midday and at a minimum in early morning.

During maturation seeds respire at a progressively lower rate; the rate

XUM

is exceedingly high in young seed. By calculating the percentage loss, based on dry weight of polysaccharides in wheat seed, the following percentage values were reached for rates of respiration over a period of 24 hr.: 21.0, 19.1, 14.5, 14.1, 13.2, 11.1, 8.3, 7.0, 6.0, 5.8, 4.5, 4.2, 3.7, 2.4, 2.2, 2.0, 1.0. Both degree of maturity and ambient temperature exert profound effects on respiration of seed (33).

Other investigators also have observed the increased respiration of wilted leaves.

Apple trees grown in pots and used to measure photosynthesis were also used to measure respiration (83). These trees, from which water was withheld, at first showed a decrease in respiration but then it rose above that of the checks, becoming 162 per cent of that of the checks. Upon supplying water to the soil, respiration gradually declined. The leaves of *Pinus nigra austriaca* (76) gradually dried in the laboratory also respired more slowly at first, but when the water content had declined to 68 per cent, the production of CO_2 was increased. In leaves dried in air of 70 per cent relative humidity, CO_2 production was depressed to a greater degree than if dried at 95 per cent relative humidity.

The leaves of tobacco (5) if removed from the plant showed increased respiration upon wilting, but after the water loss was large, respiration was decreased. A comparison of the respiration of barley seedlings, in soils of different moisture content, showed that those seedlings with the greater soil moisture had the least respiration and that the rate of this process increased as the soil moisture content was reduced. This accelerated rhythm was maintained throughout the day and night.

Desiccation of *Asparagus* stems in a vacuum accelerated respiration in proportion to the quantity of water lost.

Montfort & Hahn (70) recommended that the rate of respiration of plants be followed until they become desiccated, using this means to measure xerophytism.

The experiments described show that vegetative organs, abundantly supplied with water, react to loss of water by acceleration of respiration and of dissimilation. In some instances there is first a reduction in rate that is probably related with closure of the stomata. Then follows an increase in rate which proceeds to 160 or 170 per cent of normal. When the content of free water is decreased and only the bound water remains, respiration becomes reduced in rate.

One may readily note in mature seeds that reduction in respiration is accompanied by reduction in water content, as may be seen from the following data (33):

Water content, per cent (fresh weight basis)	67	54	50	46	36	31	26	23
Daily loss of dry weight, per cent	3.6	1.9	1.8	1.2	0.55	0.32	0.18	0.06

Osmotic Pressure

The first observations on the relation between drought and osmotic pressure were those of Pringsheim (77), using pumpkin seedlings. Osmotic pressure and resistance to drought increased in seedlings grown at low humidities.

Fitting (19) made many measurements of osmotic pressure in the Sahara desert. The species not protected against transpiration had particularly high osmotic values, that permitted them to utilize better the soil moisture. The theoretical conclusions made by Fitting have been criticized, but his observations have been confirmed by numerous investigators (4, 35, 45, 46, 63, 64). Resumes of these studies may be found in certain fundamental works (12, 66, 100). Species of the temperate zone have an average osmotic pressure of 10 atm., and those in the Arizona desert have an average osmotic pressure of 20 atm. A value as high as 50 atm. is rarely encountered, the maximum observed being that of *Atriplex confertifolia* grown on alkaline soil, which was 202.5 atm. Generally trees and shrubs have a higher osmotic value than herbaceous species. Variations occur throughout a 24-hr. period, the minimum occurring in early morning, and the maximum after midday.

When the moisture conditions are favorable for the entire plant no observable differences will be found in osmotic pressure among the leaves, stems and roots (35, 45, 46). Variations in osmotic pressure between the roots and leaves are shown in Tables II and III. These plants were grown during a humid period and a dry period in three habitats: (*a*) a marshy site, (*b*) a meadow, and (*c*) a savannah. The sites differed in moisture content of the soil and the air as shown by the measurements given below. Five typical species were examined in each locality. It may be seen that there

TABLE II

Osmotic Pressures During A Humid Period

Site	Roots	Leaves
Marsh	0.12–0.20 *N*	0.15–0.20 *N*
Meadow	0.15–0.30 *N*	0.15–0.30 *N*
Savannah	0.40–0.45 *N*	0.40–0.90 *N*

TABLE III

Osmotic Pressures During A Dry Period

Site	Roots	Leaves
Marsh	0.13–0.21 *N*	0.29–0.49 *N*
Meadow	0.26–0.35 *N*	0.32–0.65 *N*
Savannah	0.40–0.62 *N*	0.50–0.90 *N*

XUM

exist large differences in osmotic pressure between the roots and the leaves, increasing dryness being accompanied by an increase in osmotic pressure. Osmotic pressures in roots and leaves tend to be alike when there is no water deficit, but they differ greatly during dry periods. The response is greater in the leaves than in the roots since they suffer the greater loss of water. Other species studied show similar differences. Further similar differences were exhibited by potted plants.

In a series of experiments, using wheat, the soil moisture was kept favorable for best growth, and the leaves were subjected to variable contents of atmospheric moisture. As a result, the osmotic values of the roots were more or less alike whereas the dryness of the air caused a progressive increase in osmotic value of the leaves.

In another series of experiments with large pots, the moisture content of the soil was initially 72 per cent, 57 per cent, 44 per cent and 34 per cent of field capacity. The osmotic value of the roots of the wheat was 0.19, 0.26, 0.30, and 0.45 *N*, respectively, while that of the leaves varied from 0.20 *N*, under favorable atmospheric moisture conditions to 0.78 *N* under the dry conditions when the supply of water to the leaves was very limited.

Osmotic values are low in young leaves or in old leaves of a given plant when conditions are favorable for almost equal osmotic pressures throughout the plant. But when the lower leaves are completely shaded by other plants and the upper ones are exposed above the level of neighboring plants, then there is a marked difference. Thus, the lower leaves of *Lythrum virgatum* have an osmotic pressure of 0.53 *N* and the upper ones of 1.12 *N*. By the same token the osmotic values of the upper and basal parts of a leaf differ from those of the central part and the edge. In a leaf of *Typha latifolia* the part submerged in water has an osmotic value of 0.23 *N*, whereas the part just above the surface of the water has a value of 0.30 *N*, the portion 125 cm. higher of 0.64 *N*, and the part 250 cm. higher, of 0.80 *N*.

The roots respond in like manner. If the layers of soil differ in moisture content then the osmotic values are correlated with depth of soil. Not all species respond in the same way to mesic conditions. Xerophytes have higher osmotic values than do mesophytes. These differences were 0.59 *N* and 0.39 *N*, respectively in one comparison, and 0.61 *N* and 0.48 respectively in another.

The role of osmotic pressure in the life of plants is not fully understood. The osmotic values found can not save the vegetative organs from desiccation under the conditions encountered in nature. A relative humidity of 92 to 70 per cent constitutes the limit at which vacuoles of the cells of most species of plants lose all of their water, and the cells readily perish in such conditions. In Table IV are shown the osmotic values of the tissues of different ecologic types, together with the relative humidities at which the tissues perish.

The higher osmotic values favor resistance to desiccation. Another factor is the size and shape of the cells. Large cells with low osmotic values, ranging

from 0.2 to 0.3 *N*, perish in an atmosphere of 93 per cent relative humidity although small cells survive at such a relative humidity. Small cells having osmotic values within the range of 0.85 to 1.0 *N* may survive at a relative humidity of 90 to 88 per cent.

The increase of osmotic pressure can favor a better provision of soil water to the roots and also to the movement of water within the plants to parts where it is deficient. Response to drought differs among different species. Experiments by Gasser (22), using 96 species growing under conditions of drought, show that osmotic pressure increased in 69 of them, the

TABLE IV

OSMOTIC VALUES AND LETHAL RELATIVE HUMIDITIES

Osmotic values	Lethal humidities *per cent*
0.20 *N*	99
0.26–0.35 *N*	97–96
0.45–0.55 *N*	91–92
0.65–1.00 *N*	90–88

increases ranging from 15 to 2.23 per cent. Under certain conditions xerophytes can have osmotic values that are lower than those of mesophytes (28). When environmental conditions are varied a given species is able to change its osmotic value in large amount (45, 46). The osmotic value is not an indispensable criterion of resistance to drought among all species of plants. It is only one of the means of defense against drought that is inherent in each species to a different degree.

RESISTANCE OF CELLS TO DESICCATION

The principal causes of death of cells subjected to desiccation are the structure of the cells and the presence in each of a large vacuole, which conditions the contraction and expansion of the protoplast (34, 37, 40, 41, 42). If the cell is large and the vacuole is correspondingly large, and if the cytoplasm membrane is very delicate, the destruction of the cell is made easy and its resistance is lessened.

Numerous investigators have noted that species living in dry habitats have smaller cells than those living in moist habitats (49). Plants grown in a dry site tend to have smaller cells than when grown in a moist site, thus resulting in increased resistance (107).

Mosses, lichens, algae, and other lower organisms, adapted to dry sites, have cells of small volume, some being only 50 or 300 to 700 cubic microns in size. Moreover, the cell volume of higher plants is not adapted to desiccation, being 2 to 3 million cubic microns in size. Small cells when desiccated decrease their volume 1.8 to 2.0 times and large cells, 5 to 10 times. For this

XUM

reason the latter suffer large disturbances in comparison with the former. When a cell is desiccated and water is lost from the vacuole, the opposite walls approach each other; cells so treated may separate. There is greater readjustment of the walls to each other in large cells than in small ones. The shape of the cells is also a factor. The walls are farther apart in spherical and cubical cells than in elongated ones (34, 37).

The cells of certain xerophytic mosses, adapted to desiccation, are much elongated with a narrow cell cavity; their opposite walls are almost in contact. There is almost no change in the shape of such cells when water is lost from them.

In a desiccated cell, transferred to water, the vacuole imbibes water and increases its volume, the membrane stretches and the protoplast enlarges. If the tissues have not lost too great an amount of water and the protoplasm is still semiliquid, turgor is restored and the cell remains alive. As a consequence of too great desiccation the protoplasm is made dense and less fluid; its viscosity gradually increases and therefore a recovery of its initial state is very difficult. By microscopic examination, after water has entered the membrane, one may see that it swells very rapidly, but the protoplast remains contracted at the center of the cell; it produces a phenomenon known as pseudoplasmolysis. Little by little the protoplast may imbibe water acquiring a rounded form and extending toward the cell walls. This process proceeds either very rapidly or very gradually, dependent upon the degree of desiccation, sometimes requiring only a few seconds or as much as one or two and one half hours. During distention the protoplast may rupture and the sap is then dispersed, so that the cell dies as the result of moistening, not of desiccation. Rapid distention of the membrane may tear the protoplast into bits and thus cause its death. All of the phenomena, just described, may be observed very clearly under a microscope, especially in cells having colored sap (40). Marked pseudoplasmolysis was noted by Parker (74) in desiccated pine leaves that had been immersed in water.

To retard rapid distention and in this way protect the cell from killing action, one may use more or less concentrated solutions of sugars or salts, saccharose serving best for this purpose. Tissues dried in microchambers having different relative humidities may then be placed in solutions of approximately the same vapor tension as the air in the microchambers. The tissues may be transferred, at will, to more concentrated solutions or to less concentrated ones.

To dry the tissues progressively it is suggested that one commence with an atmosphere of moderate relative humidity and pass stepwise to those having a lesser relative humidity. The protoplast may be destroyed, provided the desiccating action is too drastic. By reversal of the order one may restore the water to the cells gradually after they have been dehydrated. Tissues dried in an atmosphere of 80 per cent relative humidity are killed if returned to water directly but survive if gradually passed through these solutions. By this procedure one may desiccate the cells of higher plants, having a

large watery vacuole, to a water content equivalent to an atmosphere of 50 per cent relative humidity, or even lower.

Species differ from each other markedly with respect to withstanding desiccation. The cells of some species remain alive after having been dried to a water content equivalent to an atmosphere having 50 to 55 per cent relative humidity for 2 or 3 hr., others for several hours, and others for several days or weeks. Cells plasmolyzed in saccharose solutions and stored in microchambers may exhibit a high degree of resistance to drying. Some cells can be restored after desiccation over concentrated sulphuric acid for weeks or even months, but others die after a few hours. All species of plants can withstand some desiccation, some survive severe desiccation but others are killed. They differ in respect to the duration of the dry period and the degree to which they may be desiccated. Cells having a large proportion of protoplasm and a small vacuole are least disturbed by desiccation and are protected against injury. Hibernating organs, or storage organs and reproductive structures, such as spores, zygotes, and seeds generally lack vacuoles and are filled with food reserves such as carbohydrates, proteins, and oils. In this way the species are able to survive dry periods, thus conserving life.

Buds of higher plants are very resistant to drought. Their cells are quite devoid of vacuoles. As they develop, vacuoles are formed and simultaneously resistance to drought is decreased. Germinated seeds behave similarly (66).

The vegetative organs of ferns and certain angiosperms can remain in a dry state and can then revive in water as occurs with *Nothochlaena maranthe*, *Ceterach officinarum* (78) *Larrea tridentata* (3), *Chamaegigas interpidus* (24), *Carex physodes*, *Myrothamus flabellifolia* (101), and species of *Barbecenia* (6). Two of the above-named plants have been studied as to the cause of their resistance. *N. maranthe* (39) contains cells having large vacuoles that readily absorb neutral red. The vacuoles do not become desiccated and by careful manipulation can be separated intact from the cells. Similar vacuoles are found in the cells of *C. officinarum* (78). The protoplasm of these species is protected against destruction by the firm nature of the vacuoles.

Very ample studies, made by Höfler (27) involve mosses, that he desiccated for 24 to 48 hr. in chambers having different relative humidities, and then placed in water. Each of the different species was found to have a distinct degree of resistance. Those which grew in dry sites had a very high resistance, and tolerated drying over almost concentrated sulphuric acid. Those which grew in moist habitats died after exposure to air over 5 per cent sulphuric acid. The young parts showed greater tolerance than did mature or old parts.

Studies by various workers point out the fundamental structural changes undergone by protoplasm submitted to desiccation. Viscosity is lowered (54, 81, 87), and permeability is increased (54, 81). Artificial shaking produces similar effects (48).

XUM

Based on the hypothesis proposed by Iljin that structural modification in the protoplasm arises from mechanical action, Stocker *et al.* (48, 81, 82, 90, 92) explain the sequential changes in structure of protoplasm in the following manner: They assume (21) that the protoplasm is constituted of micelles bound to each other by vacuoles. Mechanical vibration destroys the connection between micelles and lowers the viscosity of the protoplasm, increases both the size of the pores, and their permeability, and increases transpiration. The enzymes that stimulate respiration and decomposition of organic compounds are freed. Chloroplasts are injured, photosynthesis is inhibited and pH decreased. The hydration capacity is increased and the protoplasm becomes negatively charged. The decrease in pH reduces the difference of potential of the protoplasm and the micelles come closer together. Levitt (53) made a critical appraisal of this hypothesis in this journal.

Levitt also made a critical analysis of another hypothesis of drought resistance, that based upon absorption of bound water by colloids, an hypothesis maintained by several investigators (16, 72, 97, 102, 105).

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THE SUGARCANE PLANT[1,2]

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Introduction

Sugarcane, *Saccharum officinarum*, L., has been cultivated in Asia since prehistoric times. It was introduced to the New World by Columbus in 1493 and rapidly became the most important cash crop in many places [Mangelsdorf (10)]. In 1813 Sir Humphry Davy wrote in his *Elements of Agricultural Chemistry:* "The nutritive properties of sugar are well known. Since the British market has been over-stocked with this article from the West India Islands, proposals have been made for applying it as the food of cattle. . . ."

Today, cane sugar is produced for commerce in 58 countries and territories within a band around the world bounded by 30° north and south latitudes. In many other tropical places it is a garden crop. World sugar production in 1954 was estimated at over 40 million short tons of raw sugar (96 per cent sucrose) of which more than 60 per cent or about 25 million tons came from cane (8).

Per capita consumption of sugar is very unequally distributed throughout the world, ranging from less than 3 lb. per year in China to about 169 lb. in Colombia (9). The estimated 103.4 lb. consumed by each person in the United States in 1953 to 1954 comprised about 17 per cent of the total calorie intake.

Sugarcane's habit of growth which permits it to continue to store sucrose over long periods of time (two years or more) without coming to a definite maturity and death makes it one of the most efficient and productive of all crops. Spoehr (11) estimates that under optimum conditions corn can, during a four month growing period, convert about 1.5 per cent of the incident sunlight into organic matter (counting shelled corn, cobs, leaves, stalks, and roots). Sugarcane can exceed that value for short periods and can equal it over periods of nearly two years. An actual field harvest of a 22 month crop at Ewa Plantation showed a conversion of 1.43 per cent of the incident light

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XUM

recorded on a pyrheliometer. Total organic matter produced came to 28.6 tons per acre per year which greatly exceeds the best pilot plant production of *Chlorella* yet achieved [Burlew (12)]. In an interesting paper entitled "Industrialization of Photosynthesis Through the Use of Sugar Cane," Ledón & Gonzáles (13) describe cane as the most efficient of all storers of the sun's energy and review the many uses to which it can be put.

In Hawaii soil quality and climate greatly affect productivity which ranges from a maximum of about 10 tons of sugar per acre per year to one-third that amount. The "noble" varieties are so sensitive to environment that cane breeders direct much effort to producing varieties tailor-made for each locality. This task is greatly increased by the tendency of a clone to decline in yield after a few years of continuous cultivation. The problem of "yield decline" of varieties is being intensively studied in several localities. In Hawaii today 82 per cent of the total acreage is occupied by four varieties which were still in the experimental stage 10 years ago. All are descendants of H32-8560 which was the leading variety from 1941 to 1950 but is now fast disappearing.

A recent development of great interest is the characterization of "ratoon stunting disease" (RSD) as a transmittable virus. King & Steindl (14) consider this a factor in the failure of some Queensland varieties and it must be ruled out of all experiments on yield decline.

All major sugarcane-producing areas maintain experiment stations and publish one or more journals, annual reports, and special bulletins. This has built up a large specialized literature not found in the usual science journals. In the *Java Archief voor de Suikerindustrie* alone, published between 1893 and 1934 there are 40,000,000 words describing the work of the Java Proef Stations at Samarang, Kagok, and Passeroean (2, p. 587). Numerous excellent books have been written about the growing and processing of cane sugar. Nine of these have been selected as general references and are the first listings of the cited literature (1 to 9). The Library of the Experiment Station, H.S.P.A.[5], has 2886 books cataloged in the sugar section.

Growth Habit of Sugarcane

Varieties differ greatly in their growth habits and physical appearance. However, the leading Hawaiian varieties may be described in the following manner. Sugarcane grows in length about 12 ft. per year, making two to four joints per month with an average length of 4 or 5 in. Each joint has an eye which when planted as a cutting, develops into the primary shoot; and a root band from which come "cutting roots". These temporary roots decay after the shoots have established their own permanent shoot roots. Stalk diameters usually range between 1 and 2 in. For details of sugarcane anatomy see Martin (7) and Dillewijn (3).

Tillering or stooling gives rise to secondary and tertiary stalks. The term

[5] H. S. P. A., Hawaiian Sugar Planters' Association.

"sucker" is sometimes applied to stalks appearing in the second season of growth. A stool, starting from a single eye, when free from competition, may expand to several feet in diameter in 18 months. For example, Shamel (15) pictures a Uba single-eye stool with 144 stalks weighing 334 lb. after stripping the dry leaves (Fig. 1). In Hawaii a two year crop at harvest time consists of stalks of all possible ages, some dying from competition in their old age. A few stalks attain the extreme length of over 40 ft. in this period.

After harvest the ratoon or stubble crop rapidly develops the new shoots from underground buds, many of which die out from competition. Ratooning may continue for several crops depending upon the nature of the soil and its freedom from compaction. With three ratoons in Hawaii the crop cycle is eight years between plantings. The long cropping of Hawaii is exceptional. In most of the cane-growing regions crops are harvested after 12 to 14 months.

Fifty thousand stalks per acre comprise a dense stand of cane. Each stalk carries eight to twelve leaves, each of which consists of a sheath and blade. The sheath is attached to the stalk node and joins the blade at the leaf joint or collar (dewlap). A 12 in. sheath may carry a blade 48 in. long of 2 to 4 in. maximum width.

An average of about three leaves unrolls per month. For easy convenient reference they are numbered from the youngest downward. Hence all leaves and nodes of the same number are about the same age.

Flower initiation occurs at the terminal bud, the upper embryonic joints elongating greatly into the flower stalk which may be 4 or 5 ft. long. This permanently stops terminal vegetative growth and releases the lateral buds from hormone inhibition. Usually the top four buds start growth almost immediately, producing branches or lalas. These branches may produce many feet of mature cane the following year.

Also following floral initiation is a marked shift in growth of the young leaves immediately below the flower bud. Sheaths grow longer and blades shorter, with combined length remaining nearly constant, until there is a blade only a few inches long atop a sheath several feet long. This "flag" predicts early emergence of the tassel.

Composition

The composition of sugarcane is of importance in technology because of the potential by-products available after separation of the sucrose. Its nutritional values are of interest to millions who use it as a garden vegetable known as "chewing cane."

For each ton of sucrose produced from a well-matured crop there is about 1 ton of bagasse (fiber), 1/4 ton of molasses, 20 lb. each of wax and aconitic acid. These materials assume commercial importance because they accumulate at the sugar mill in sufficient quantities for industrial plants of economic size.

Bagasse is finding a wide and profitable use in paper making, wallboard, bedding for animals, mulch and organic matter for soils, in addition to being

XUM

the sole fuel for most sugar mills. Molasses is used chiefly as animal feed and as the nutrient for alcoholic fermentation. When in excess it is returned to the soil as fertilizer. The wax is of good quality for polishes and the aconitic acid goes into plastics. The sterols are of potential interest to organic chemists.

Fiber.—The sugarcane plant usually contains from 6 to 14 per cent fiber (16, 17, 18). According to Roza (17), this is composed of Alpha cellulose (34.7 to 37.7 per cent), pentosans and other sugars (21.4 to 29.1 per cent) and lignin (18.2 to 26.6 per cent), with smaller amounts of Beta and Gamma cellulose. Wiggins (19) gives 50 per cent cellulose, 25 per cent each of pentosans and lignin as average figures.

Steuerwald (20) isolated an aromatic fraction of lignin for which he coined the much-quoted name "saccharetine." It contains pyrogallol, protochatuic acid, and vanillin. This may be the same as the "tannin" of Zerban (21).

De Stevens & Nord (22) characterized sugarcane lignin as being similar to that of maple, classifying it as a "guaiacyl-syringyl lignin."

The hardness of the rind of sugarcane has been measured with penetrometers by Puri (23), who found 2.5 to 10.3 lb. needed to pierce the rind. A similar variation, 3.9 to 17 lb., was found by Pemberton (24), tasseled stalks being regularly much softer than nonflowering stalks of the same age. The hardness is positively correlated with the amount of lignin in the stalk, but not with silica (25, p. 24).

Phloroglucin was found in K-deficient cane by Hartt (26), who considers this a decomposition product of tannin. Hartt also found calcium pectate (27). Farnell (28) found 0.00 to 0.015 per cent calcium pectate in juice from Trinidad sugarcane, but 0.13 per cent in a sample from Demerara.

Shikimic acid is probably an intermediate in the formation of lignin, according to Eberhardt & Schubert (29).

Vitamins.—Nelson & Jones (30) report the presence of Vitamin A in sugarcane juice, but their evidence is not conclusive. They also note the absence of Vitamin D. The work of Jackson & Macek (31) gives the following ranges, expressed as micrograms per gram of cane stalk: thiamine HCl, 0.19 to 0.79; riboflavin, 0.13 to 0.40; pantothenic acid, 0.54 to 4.27; niacin, 0.85 to 3.03; biotin, 0.01 to 0.11.

Pyridoxin to the extent of 10 μg. per 100 gm. cane leaves was found by Inagaki & Nagao (32). Binkley *et al.* (33) report 0.005 per cent inositol in juice.

Binkley & Wolfrom (34) give a value of 0.43×10^{-5} per cent folic acid in molasses. A statement (35) that "sugarcane molasses is the richest natural source" of folic acid is simply a misquotation of Hauser (36).

Ascorbic acid has been determined by Munsell (37) as 0.5 mg. per 100 gm. juice, and by Wasicky & Ferreira (38) as 12 mg. per 100 gm. leaves.

Although Payne & Gill (39) found no choline in their sample of molasses, a figure of 290 mg. per lb. is widely used commercially (40, for instance).

FIG. 1. Stool of Uba cane grown from a single-eye cutting. The 144 stalks weigh 334 lb. [after Shamel (15)].

There may, perhaps, be confusion with molasses from sugar beets, which does contain this compound.

Lipides.—Most work on the lipides of sugarcane—commonly called "cane wax"—has been done by extracting residues after processing with organic solvents. This "wax" amounts to .05 to .5 per cent of the cane (19, 41, 42). According to Freeland (41), 30 to 50 per cent of this is the hard "wax" from the exterior of the cane stalk. Kapil & Mukherjee (43) report this to consist mainly of myricyl alcohol (36 per cent) and hydrocarbons (52 per cent).

Mitsui & Matsuda (44) and Whyte & Hengeveld (45) published fatty acid analyses of crude extracts, whose major constituents are palmitic and linoleic acids. Wagtendonk & Wulzen (46) isolated a compound amounting to 0.6 per cent of the crude wax, which was positively identified as stigmasterol by Kaiser & Wulzen (47). Balch (48) also identified stigmasterol, as well as sitosterol and carotene. Chlorophyll and glycerol are mentioned by Binkley & Wolfrom (49). Honig (50) found .012 per cent lecithin in cane juice.

Organic phosphorus.—The phosphorus compounds of sugarcane have not been thoroughly investigated. Wiggins (51) identified glucose-1-phosphate, Honig (50) lecithin. Using radioactive tracers, Burr & Kortschak [52, 53 (p. 14)] identified fructose diphosphate, glucose-1-, and glucose-6-phosphate, adenosine triphosphate, phosphomalic acid, and 3-phosphoglyceric acid. The presence of this last is noteworthy in that the authors [54 (p. 10), 55] have shown that in the sugarcane, photosynthesis proceeds through phosphomalic acid rather than through phosphoglyceric acid (PGA) as in the algae.

Binkley *et al.* (33) found .186 per cent phytin in molasses, but Payne & Gill (39) found none in their sample.

Organic acids.—Except for the commercially important aconitic acid, knowledge of the non-nitrogenous acids of sugarcane rested on the classic paper of Yoder (56) until the recent work of Roberts *et al.* (57, 58, 59). In dry juice solids they found (59) .43 to 2.07 per cent aconitic, .02 to .22 per cent citric, trace to .02 per cent fumaric, .01 to .14 per cent glycolic, .12 to .42 per cent malic, .02 to .06 per cent mesaconic, .03 to .17 per cent oxalic, .01 to .06 per cent succinic, and a trace of syringic acid. It is striking that in one case (58) they found more both of mesaconic and succinic acids than of aconitic. On the other hand, Balch (60) found a sample of "luxuriant" cane containing 13 per cent aconitic acid in juice solids. As regards oxalic acid, Bosz (61) found most to be insoluble; this would not be found by the methods of Roberts *et al.* Formic acid (62) and 2-keto-gluconic acid (51) have also been found, probably due to fermentation.

Nitrogen.—Total nitrogen of the cane plant varies greatly with amount of fertilizer, age and variety. Cornelison (63) has shown that nitrates are absent in sugarcane, all nitrogen being present as ammonia or in organic form. Leaf nitrogen is largely protein (19, 64, 65) but in the stem nonprotein nitrogen may predominate (66, 67). According to Wiggins & Williams (64), the same amino acids occur in the stalk juice as in the leaves. These authors found a spectacular increase in the amount of amino acids (up to 10 times) when the cane suffered from drought.

Qualitatively as well as quantitatively, the amino acid composition is extremely variable, as shown by the analyses by Kelly & Thompson (68) and Wiggins & Williams (69). Although asparagine is frequently the amino compound present in largest quantity (67, 69, 70), Wiggins & Williams found it absent in at least one sample. Parish (71) found six varieties to be much alike in amino acid content.

Except for the identification of asparagine in juice by Hardy (67), and of aspartic and glutamic acids and lysine by Payne & Gill (39) in molasses, all amino acid analyses of sugarcane have been by paper chromatography. Identifications of individual compounds by this method are subject to considerable uncertainty, and are always dependent on the availability of known reference compounds.

All these analyses agree in showing the presence of aspartic and glutamic acids, alanine and valine (55, 64, 68, 69, 70, 72, 73, 74). Wiggins *et al.* (64, 69, 72, 74) also found γ-amino butyric acid, lysine, leucine, glycine, serine, glutamine, and phenylalanine. Norleucine, tyrosine, cystine, and threonine were identified in Queensland (73), isoleucine by Kelly & Thompson (68), methionine, norvaline, and α-amino isobutyric acid by Kortschak (55).

In molasses, Payne & Gill (39) identified guanine, 5-methyl cytosine, and (probably) proline.

Auxin.—Brandes & Overbeek (75) found 1.4 to 4.1 mg./kg. free and 2.5 to 8.3 bound auxin in sugarcane nodes. The work of Engard & Larsen (76) makes it probable that this is indole-3-acetic acid.

Mineral Nutrition

Commercial varieties of sugarcane differ greatly in their ability to extract nutrients from soils and in their content of mineral elements. Therefore, statements about nutrient requirements and composition must necessarily be general. When specific cases are cited it is understood that they apply only to the described conditions.

Record crops of sugarcane produce 50 tons of harvested dry matter per acre in less than two years. This well-fed cane removes as much as 300 lb. N, 80 lb. P and 800 lb. K according to Borden (77). Other elements are removed in proportionately large amounts and it is not surprising that deficiencies of several have been reported. Martin (7) describes the deficiency symptoms due to lack of B, Ca, Fe, Mg, Mn, N, P, K, and S, when produced in culture solution. Eight color plates illustrate the visible leaf abnormalities. Except for nitrogen, deficiencies in the field are not commonly severe enough to produce leaf symptoms even though yield is reduced.

All sugar fields in Hawaii require nitrogen additions for maximum yields. Many require P and K. A few require Ca (as an essential element) and some are quite low in Mg. Iron chlorosis is commonly observed in young cane, especially in ratoons. This condition is alleviated by iron sprays but the treatment is not commonly applied commercially since the symptoms disappear with age. Sulfur deficiency has not been seen in Hawaiian fields. Pahala blight (7, p. 95) proved to be due to Mn deficiency. Other minor essential elements (B, Zn, Cu, and Mo) have not been found beneficial in the cases where their deficiency has been suspected. In fact it is difficult to produce their deficiency symptoms in culture solution (78).

In a rather thorough survey of the sugar fields of British Guiana, Evans (79) finds areas in which there is the possibility of a deficiency of B, Cu and

Mo. However, none have been shown to be of great economic significance there.

In Puerto Rico, Samuels, Lugo-López & Landrau (80) found that fertilizer containing the minor elements Cu, Mg, Zn, B, Mn, Ca, Fe, and S did not improve yields.

Diagnosis by tissue analysis.—The effort to maintain maximum yields for survival in the highly competitive sugar market has led to extensive use of plant analysis for early detection of nutrient deficiencies. Systems of "foliar diagnosis" or "crop logging" differ considerably in choice of tissue and estimates of "critical" levels of individual elements. The long-cropping of most sugarcane (12 to 48 months) poses at least two questions to be answered by the physiologist: (*a*) the adequate nutrient level for optimum growth *at the moment* and (*b*) prediction of needs for the future to avoid (i) running out too early or (ii) an excess at harvest time which may lower sucrose content and juice quality. Early running out of nutrients may be avoided on irrigated plantations by additions of fertilizers to the water. And, with the advent of the agricultural airplane, additions by air can be made to all but the most rugged areas after the cane has "closed in."

The first systematic study of foliar diagnosis in Hawaii was made in 1939 by Yuen & Hance (81). Methods and estimates of adequate levels of nitrogen obtained by analysing disks punched from leaves are given. Over a considerable period of years data have been collected in Jamaica on the relation of N, P and K content of the lamina to yield. Innes & Chinloy (82) find the data relating to potash by far the most accurate and those relating to nitrogen the least satisfactory. They state, "leaf nitrogen cannot as yet be used to predict quantitative responses to nitrogen." They resort to differential treatments of microplots within a field for the best estimate of nutritional status.

The extremes in philosophy of feeding and diagnosing the well-being of sugarcane are represented by a system widely used in Hawaii and one in Mauritius. Clements (83) begins analyses at two to three months of age and continues sampling every 35 days through the life of the crops. Nitrogen is applied in small increments (40 to 60 lb./A.) on "the principle of multiple doses versus a single dose." He continues "each of the several applications we make has a function to perform." A total of four or five applications are made in the first year of the crop; the timing and amount are controlled by the leaf punch nitrogen, assuming a "critical level" is known.

On the other hand, 20 years of experience in Mauritius has led Halais (84) to conclude: (*a*) that all fertilizer should be applied early; and (*b*) that extraneous influences make it impossible to designate a "critical level" for N. The recommended procedure is to apply the estimated "field practice" fertilizer at the beginning of the crop and at about three months of age add an extra 40 to 50 lb. N/A. to 6 to 12 microplots well scattered through the field. One and two months later leaf samples are taken. If the Vegetative Index has increased to 1.05 and the leaf N is 0.1 per cent above the field average, immediate application of more fertilizer to the field is recom-

mended. The principle of leaf saturation with nitrogen at an early age is applied.

Actually there is little evidence for the need for late application of fertilizers except on the most highly leached thin soils. In Mauritius, Evans (85) states that the best effect comes from nitrogen when all is added at planting. This is supported by the most recent evidence (86) from 81 trials which were located in three climatic zones, although there is a hint that in some regions a later application of nitrogen might be beneficial.

In Puerto Rico, Samuels (87) finds that samples taken at three months of age give the best correlation between nitrogen content of the leaf and cane yields per acre. He hopes to eliminate second applications of fertilizer by using data from previous crops. Even with the long cropping in Hawaii there is no clear evidence supporting the need for late fertilizer applications (88, p. 9). In fact recent experiments (25, p. 10–11) show that 200 lb. N/A. is required in the first three months to give maximum stand of large stalks.

There is a widespread feeling that age, cane variety, temperature and moisture so affect nitrogen composition that it is impossible to set a "critical level" for N, (84, 89, 90, 91). Therefore the relative changes due to increments of fertilizer within a field have been adopted by some as the most reliable index.

In addition to chemical analysis other indices of the general well being of the cane have been adopted. Notable among these is the Vegetative Index (weight of 30 blades) of Halais (84) and Rouillard (92). The latter suggests that leaf-weight index is such a reliable measure of sugar yield that harvesting experimental plots is unnecessary.

In most localities young leaf blades have been selected for analysis. In Hawaii, Clements (93) uses the young blades and sheaths. The authors (94 to 97) have found that stalk analyses give valuable information about the nutritive state of the plant. Some analytical values associated with optimum yields are given by Burr (98).

Potassium.—Many workers have described symptoms of potassium deficiency in plants grown in culture solutions, e.g., Hartt (26, 27), Honert (99), Suzuki & Kenjo (100), and Blackburn (101), the main symptoms being retardation of growth, dieback of older leaves and drying of leaf margins, spotting of leaves, and red coloration on upper surfaces of midribs of older leaves. Similar symptoms have been noted in the field, e.g., in South Africa [Lintner (102); Du Toit (103)], in Mauritius [Feillafé (104)], and in Formosa [Saito (105)].

Very interesting studies on root exudates were conducted by Evans (106) who reported that the exudate from deep roots always contained more K than that from superficial roots. The author was not sure whether these results indicated relative absorption by the different classes of roots, or relative storage. Our studies of the absorption and translocation of radioactive rubidium (as K indicator) and other tracers, all show complete mixing in a circulatory system, which would indicate that the composition of root ex-

udates cannot be taken as a measure of absorption by particular roots, but rather as a measure of their ability to remove elements from the common pool.

The distribution of potassium in the plant was studied by Ayres (107) who reported greatest content of K in the top 6 inches of stalk (growing point), decreasing down the stalk at all ages. The percentage of potassium in the stalk decreased with age of plant. Green parts of sheaths had higher percentages of potassium than green blades. The percentage of potassium in the leaf decreased with age of leaf. Kenjo (108) studied potassium distribution in leaves and found most of the potassium in the lower part of a leaf, decreasing gradually to the tip; and also stated that the lower leaves of plants supplied with or deprived of potassium differ more in percentage of potassium than the upper leaves. Burr & Tanimoto (95) reported that the concentration of potassium in well-fed and deficient plants differed more in the old cane and roots than in young growing tissues; very young blades and sheaths showed very little difference, while the response increased steadily down to the 9 and 10 leaves.

Du Toit (103) stated that potassium deficiency in South African soils is invariably accompanied by abnormal accumulation of magnesium and sometimes calcium. Evans (109) found a very high correlation between potassium and nitrogen in all parts, but Clements *et al.* (110) found total N lower in the potassium deficient plants than in the controls, in the dry-leaf cane, with irregular results in the rest of the plant. Hartt (26, 96) found that plants deficient in potassium absorbed more phosphorus and iron than the controls, also more calcium, magnesium, and silicon during the first two to three months. Plants with low supplies of potassium absorbed more phosphorus than the controls and as a result their leaves contained more phosphorus, especially inorganic phosphorus. Suzuki & Kenjo (100) reported that leaves of potassium-deficient plants contained much more total N, Ca, Mg, P, and S than controls. Plants deficient in potassium had a decreased percentage of moisture and decreased rate of transpiration, according to Hartt (26, 27) and Humbert (111) stated that with subnormal potassium it was practically impossible to get adequate moisture into the plant.

More reducing sugars and less sucrose were found in the blades and tops of plants deprived of potassium than in controls [26, 112 (p. 44)]. But stems or juice of deficient plants had less reducing sugars and sucrose, in the report of Suzuki & Kenjo (100) and Hartt (26). Cause of the higher reducing sugar/sucrose ratio in plants deficient in potassium is not known. Detached blades showing symptoms of potassium deficiency made less sucrose than control blades, when supplied with glucose in the dark.

Plants deficient in potassium had more amino N than the controls, according to Hartt (26), and in another test at the H.S.P.A. (112, p. 44) low potassium more than doubled the amide N in tops above the fifth joint harvested after four months.

Phosphorus.—Symptoms of phosphorus deficiency in sugarcane have been described by workers in many sugar-producing areas, including Honert

(99), Martin (7), Saito & Kenjo (113), Rege & Sannabhadti (114), Clements *et al.* (110), Bourne (115). Phosphorus deficiency results in retardation of growth, lack of tillering, reduction in both length and width of leaves (effect more pronounced on width), and in length and diameter of internodes. Roots are longer but more slender, red-brown in color, and subject to rot. Young leaves show little difference in color; are sometimes greenish-blue; but old leaves turn yellowish-brown and dry and die at tips and margins, but show no spotting or striping.

The rate of absorption of phosphorus by sugarcane is affected by reaction of solution. Schroo (116) reported that maximum absorption took place at pH 4.5, 83 per cent at pH 6.0, 33 per cent at pH 7 and 14 per cent at pH 7.5, but in the presence of Fe, Al, Mn, and Ti ions, the pH values for uptake of phosphorus are different. Suzuki & Kenjo (117) stated that more phosphate was absorbed when the nitrogen source was nitrate than when ammonium was used, probably connected with differences in hydrogen ion concentration. Plants of the same size absorbed phosphorus from nutrient solutions at the same initial rates, regardless of amount of phosphorus in the nutrient solution, in studies reported by Hartt (96).

Temperature also affects absorption. Low root temperatures cut down absorption to the same extent as growth, resulting in nearly uniform percentages of phosphorus in the plants (96). Root temperature may have affected absorption directly since the effect was apparent from the start. Air temperature, however, affected the size of the plant primarily, and the smaller plant at lower temperature absorbed less phosphorus in recent studies at the H.S.P.A. (55).

The distribution of phosphorus in tissues of sugarcane was studied by microchemical methods by Lazo & Bonazzi (118), who found that phosphorus was present principally in the phloem in the stem, being mainly in the nuclei of companion cells; the sieve tubes were comparatively weak in phosphorus. In the leaf, the greatest amount of phosphorus was in the chlorophyll-bearing sheath of the vascular bundles. The young, white leaves in the spindle were very rich in phosphorus. This very interesting study showed the phosphorus topography coinciding with centers of greatest activity of growth and assimilation (meristematic and photosynthetic tissues), but unfortunately the root was not included in the study.

The distribution of phosphorus in the plant was studied by Evans (119) who reported generally more phosphate in leaves than in stems or roots, the total phosphorus per stool increasing to the end of the experiment. Ayres (107) reported the percentage of phosphorus in the plant decreased with age, more slowly in leaves than in stalks. Highest concentration of phosphorus was found in the growing point. Clements (120) made thousands of analyses on different parts of the plant at different ages, both in the field and in nutrient solutions with and without phosphorus. These he correlated with growth rates from which he was able to make recommendations for fertilization.

The percentage of phosphorus is decreased by high amounts of K, Ca,

XUM

Mg, and S [Hartt (121)], and Borden (122) found decreased percentages of phosphate whenever the percentage of nitrogen was increased. A strong depressing effect of nitrogen on stalk phosphorus was reported by the H.S.P.A. (25, p. 10–11).

Analyses of inorganic and organic phosphorus in leaves were performed by Hartt (96). Inorganic P was most closely responsive to the amount of phosphorus supplied in the nutrient solution or in the soil. In luxury feeding of phosphorus, it is the inorganic form that accumulates.

Rege & Sannabhadti (114) stated that phosphorus maintains the water content of the leaf at a high level, and accelerates the uptake of N, K, and Ca, and the rate of photosynthesis. Saito & Kenjo (113) found the ash of phosphorus-deficient plants high in Si; the roots were very high in Fe; all parts were high in N. But Clements (110) found no regular effect of phosphorus on total N, K_2O, or Ca.

Three months after planting 85 per cent of the P^{32} in seedpieces was found distributed throughout the young plant (53, p. 12).

Translocation of P and K.—Since the lower leaves of a sugarcane plant often contain less phosphorus and potassium than the upper leaves of the same plant, these elements were thought to move out of the older, dying leaves to be reutilized in the younger leaves, according to Ayres (107), Hartt (26), Suzuki & Kenjo (100), and Kenjo (108). The greatest migration of potassium occurred in the blades with the lowest moisture percentage (26). The author concluded that translocation of potassium is not an adaptation in potassium-deficient plants. Suzuki & Kenjo (100) stated that older leaves always had less potassium than younger leaves, without reference to a deficiency in potassium. Kenjo (108) reported that migration of potassium took place in plants deficient or sufficient in potassium, but found relatively more migration in potassium-deficient plants. We now know from our tracer studies herein reviewed, that phosphorus and potassium, as well as nitrogen and carbon, are continuously circulating throughout the plant, each part taking from the common pool what it can use for growth and other physiological processes. Potassium and phosphorus are moving out of and into every leaf all the time, the percentage composition at any time being indicative of the condition of vigor of the leaf.

Carpenter (123) grew sugarcane plants in phosphorus-deficient nutrient solution with one root in a vessel containing sodium phosphate only. Root growth and condition indicated that phosphorus was assimilated by the one root benefiting the entire plant, while the other essential elements absorbed by the root mass as a whole nourished the isolated root.

Studies of the absorption and translocation of radioactive phosphate have been reported by the H.S.P.A. [124 (p. 10), 125 (p. 9–11), 53 (p. 12–15), 55], Burr *et al.* (126), and Hartt (96). When P^{32} was fed into a culture solution, radioactivity could be detected well up the stalk within a half hour, and after one hour the P^{32} was distributed uniformly throughout the leaves. Two months later, new blades were more radioactive than old, and recently

formed branches and suckers were as rich in P^{32} as the parent plant. So there is a pool of phosphate on which the entire plant can draw.

Field studies of placement of phosphate have shown the importance of getting the phosphorus into the root zone. Best position was beneath the cutting or directly under the old plant. Schroo (116) stated that a sugarcane plant is a number of individual stalks, each fed by its own root system "without any inter-communication," a statement not in agreement with Burr (127) who reported that all our experiments with tagged elements have shown continued mixing through a common pool. We have recently definitely proved interstalk translocation of P^{32} in two tests, by injection into the stem and by application to a leaf (55).

The translocation of potassium has been studied using rubidium 86 as indicator, a method adopted because of the short half-life of K^{42}. Rb^{86} was found to follow potassium quantitatively in tissues of sugarcane, potassium being determined with a flame photometer and Rb^{86} by Geiger counting, in studies by the authors [53 (p. 13), 95, 126, 127, 128 (p. 9)]. When Rb^{86} solution was applied to one leaf, it slowly entered the circulatory stream and migrated to all parts, including young sprouts. Rb^{86} required 15 days for half-absorption by a leaf. When the rubidium reached the stalk, most of it turned upward into the young leaves.

Translocation of N^{15} ammonia and urea.—Studies of root feeding of N^{15} ammonia and leaf feeding of N^{15} urea by the authors [53 (p. 8–11), 55, 88 (p. 9–10), 94, 112 (p. 43–45), 125 (p. 12–14), 127] have shown that tagged N is quickly absorbed and translocated to all parts of the plant and into all chemical nitrogenous fractions.

Leaf-punch studies showed that new nitrogen absorbed by the roots spread rapidly in the plant, going in greatest amount to the leaves already richest in nitrogen, probably because of their high metabolic activity. The entire nitrogen content of the plant is a pool upon which all parts draw according to their needs and capabilities. Nitrogen is in a continual state of flux, circulating from primary shoots to suckers and back again. More vigorous suckers slowly rob primary shoots of available N.

When sugarcane plants were fed N^{15} ammonia via the roots, the amide N fractions were the richest in new nitrogen. Proteins contained relatively small amounts of new nitrogen, but even in the oldest joints some new nitrogen was incorporated into protein. Asparagine, glutamine, and ammonia are the mobile nitrogen fractions in sugarcane. It is interesting that asparagine is an early product formed from tagged ammonia and aspartic acid is an early product tagged with C^{14} in photosynthesis.

N^{15} urea sprayed onto a sugarcane plant was rapidly absorbed. Some tagged N moved to new tissues but most of it remained in the sprayed leaves.

Tagged urea applied to dewlaps of leaves four and five of several single-stalked plants rushed first into the laminae of the fed leaves and later became redistributed throughout the plant.

XUM

Carbohydrate Metabolism

Photosynthesis.—Rates of absorption of CO_2, were studied by Singh & Lal (129), Singh (130), and by Lal & De (131). At all ages of the plant, real assimilation was greatest in young leaves, less in mature, and least in old leaves. Since the external conditions of CO_2 concentration, temperature, and light intensity were controlled, these differences in assimilation were due to internal factors the exact nature of which was not found. Differences in rates of assimilation were detected before differences in growth were observed, in plants grown with several amounts of nitrogen, phosphorus, and potassium added to soil cultures. Assimilation of CO_2 was increased by nitrogen and decreased by phosphorus fertilization in leaves at all stages; while potassium increased assimilation by leaves during the first half but decreased it during the second half of the growth period of the plant.

The greater photosynthetic activity of young than old leaves was also found in defoliation studies by Yamasaki & Arikado (132, 133). They used the half leaf method to study assimilation in different seasons and found maximum assimilation in September and believe there may be a relation between the formation of flower buds and high intensity of carbon assimilation.

Studies with radioactive carbon dioxide at this Station [54 (p. 10), 112 (p. 44)] used in the field to test photosynthesis as affected by moisture and nutrition, have shown that the uptake of $C^{14}O_2$ was reduced by severe nitrogen deficiency to one-fifth that of the controls.

A fundamental study of the effect of light, concentration of CO_2, and temperature by Singh & Lal (134) illustrated the interrelationships of these factors. At any concentration of CO_2, assimilation was positively correlated with light intensity. The higher the light intensity, the higher the optimum CO_2 concentration. With 0.05 per cent CO_2 and 1875 c.p., maximum assimilation was obtained at 34°C. They (129) also determined the rate of assimilation at the mean CO_2 concentration of a sugarcane field (.061 per cent CO_2) compared with .33 per cent CO_2. At the higher concentration there was a characteristic optimum photosynthesis at 220 days of age.

Water supply materially affects rate of photosynthesis. Although some photosynthesis did take place in leaves of plants at or below the wilting point, as shown by gains in sugars during the day, the amount of photosynthesis was considerably less than in plants adequately supplied with water, according to Hartt (135, 136, 137). Recent studies here with a continuous recording infrared spectrophometer have shown that photosynthesis falls to about 25 per cent of normal when soil moisture drops to permanent wilting point [25 (p. 14), 54 (p. 17–18), 138].

A depression in rate of photosynthesis at midday was detected by Yap (139), McLean (140), Gooding (141), but Ashton (138) reported a midday recovery of photosynthesis in a plant under moisture stress, attributed to shading of lower leaves by the upper ones. Singh & Singh (142) found that glucose and sucrose increased until 2 P.M. and starch until 10 P.M., in attached leaves. Hartt (143) obtained increases in sucrose until 5 or 6 P.M., starch and

other polysaccharides until 7 P.M., followed by rapid decreases during the night.

Studies designed to determine the intermediates found in sugarcane by tracer techniques with C^{14} and P^{32} have been summarized by Hartt & Burr (144), and Baver (145). Unlike algae and some other plants used by Calvin and co-workers, in sugarcane C^{14} is detectable in free sugars before the phosphorylated ones and in glucose and fructose before sucrose (88, p. 47). Although detached blades supplied with glucose or fructose (either radioactive or not) in the dark, transformed the reducing sugars to sucrose (112, p. 43), we now know that in photosynthesis glucose and fructose increase along with sucrose, and are all therefore classified as end products rather than intermediates (146).

Phosphoglyceric acid has been found by Burr & Kortschak (52) as a regular constituent of sugarcane leaves and juice expressed from stalks. However, in very short-time tests of steady-state photosynthesis with C^{14}, it is not detectable among the labeled compounds (54, p. 10). Malic acid, phosphomalic acid, aspartic acid, and several as yet unidentified compounds account for a large per cent of total counts, and these counts find their way into the three sugars (primarily sucrose) when washed out with C^{12} (146).

Sugar transformations.—The transformation of sugars to starch was studied by Coelingh & Koningsberger (147). De-starched leaves formed starch when floated on solutions of sucrose, maltose, glucose, or fructose. The starch so formed was located chiefly in the palisade parenchyma, although the starch formed in photosynthesis was located chiefly in the starch sheath. Starch formed from sugars in the dark and stored in the palisadeparenchyma, was converted and formed anew in the starch sheath under the influence of light. The formation of starch from sugar was increased by light, decreased by low temperature, and did not require chlorophyll. Balch & Friloux (148) state that starch in cane juice is high as long as the cane is physiologically active. It serves as a reserve food.

The interconversion of glucose and fructose, the formation of sucrose from reducing sugars, and the transformation of sucrose have been studied by Hartt (112, 149, 150, 151). These processes were found to take place in all organs of the sugarcane plant, blades, sheaths, stems, and roots when supplied with sugars. Neither light nor chlorophyll was needed for the interconversion of glucose and fructose or for the formation of sucrose, but aeration was essential. The processes were considered to require phosphorus, increased to a maximum with rising temperature, and continued in the same detached blade for two weeks. Many factors were found to decrease the formation of sucrose but the only factors which consistently increased the formation of sucrose were a rising temperature, improved aeration, and the addition of malic acid. Studies with inhibitors led to the suggestion that fructose diphosphate and sucrose phosphate were intermediates in the transformation of reducing sugars to sucrose. When radioactive invert sugar was fed to detached blades in the dark, radioactive sucrose was

XUM

formed. Therefore the sugarcane plant possesses in all four organs a mechanism for the direct or indirect formation of sucrose from glucose or fructose; but this mechanism is not the only, or even the main, pathway to sucrose, for our photosynthesis studies using tracer carbon have shown that glucose, fructose, and sucrose all increased in radioactivity together. If glucose and fructose were converted to sucrose in photosynthesis, as they are in dark transformations, labeling in the reducing sugars would decrease as it increased in sucrose, when washed out with C^{12}, which is not the fact.

Aerated blades supplied with sucrose gained chiefly sucrose, transforming only a small percentage to reducing sugars, chiefly fructose. Another product obtained was water-soluble, was not hydrolyzable by Taka-diastase but hydrolyzed by acid to form glucose, as reported by the authors (152).

The formation of sucrose by blades dipped in solutions of simple sugars has also been studied by Sen (153) who found greater absorption in light than in the dark.

Enzymes.—Invertase was studied by Hartt (121) who found its activity affected by mineral nutrition, increased by potassium, calcium, nitrogen, and phosphorus but decreased by magnesium. Fujii (154, 155) reported maximum activity of leaf invertase at 35°C., pH 5.2 and sucrose concentration below 5 per cent; also reported the distribution of invertase in various parts of a leaf and in leaves in different positions on the plant. Other carbohydrate enzymes studied include amylase, dextrinase, and maltase (121).

One of the most interesting recent studies of enzymes in sugarcane was that reported by Cardini *et al.* (156). They suggested the name "saccharese" for an enzyme prepared from shoots and roots (but not leaves), which catalyzes the reaction: UDPG+fructose=sucrose+UDP.[6]

Respiration.—The importance of respiration in the sugarcane stem was illustrated by Hes (157) who found values ranging from 2 gm. CO_2/kg. dry wt./24 hr. in the lower part of the stalk to 13 gm. CO_2 in the top of the stem. The latter value corresponds to the dissimilation of about 9 gm. hexose/kg. dry wt./day. Calculated on the acre basis this would represent a large amount of photosynthate used up for energy alone. Respiration of young leaves of eight varieties differing as to drought resistance, was studied by Khanna & Raheja (158, 159) who obtained a fair correlation between magnitude of depression in respiratory rate and drought resistance of the varieties. Maximum respiratory rate was obtained at 38°C. in the laboratory. Fluctuations in rate in the field during the day were mainly connected with temperature. Varietal differences in respiration were connected with amounts of chlorophyll. Irrespective of variety, season, and time of day, respiration decreased with age of plant (159).

Lal & Srivastava (160) measured respiration of stems at 31°C. Intensity of CO_2 output declined with each successive increase in age of tillers,

[6] UDP, Uridine diphosphate; UDPG, Uridine diphosphate glucose. The uridine phosphates resemble adenosine phosphates.

and was most active at the top of each class of tillers. Respiration was positively correlated with glucose and negatively correlated with sucrose.

Respiratory enzymes were studied by Bonazzi (161) who located oxidases chiefly in meristematic tissues, by the guayac test. Hartt (121) reported that phosphorus increased the activity of oxidase, while potassium and nitrogen each increased the activity of catalase.

Absorption of carbohydrates.—The sugarcane plant can absorb soluble carbohydrates through the roots, stems and leaves. The absorption of glucose by detached roots and blades was increased by aeration and temperature [Hartt (150, 151)]. Radioactive sucrose painted on a blade surface entered the leaf rapidly, about 50 per cent the first day, in a study by the H.S.P.A. (125, p. 9–10).

Translocation of carbohydrates.—Water is important in determining which sugar is translocated the more predominantly in a given plant at a given time [Hartt (135)]. The same author (162) showed that the transport of sugar occurs both day and night. The temporary storage of polysaccharides in the sheath during the night aids in the transport of sugar from blade to stem. Potassium deficiency was found to interfere with the translocation of both carbohydrates and proteins (26).

Translocation of C^{14}.—Several experiments on the transport of radiocarbon compounds formed by photosynthesis in a leaf have been reported by the authors [55, 88, 124 (p. 55–56), 126, 127, 144, 151, 159, 163]. Radioactive compounds formed in one leaf of a single-stalked plant were distributed to all parts of the 11-ft., 7-lb. stalk within three days. In another experiment material made by the upper half of a single blade fed radioactive carbon dioxide was respired by the roots 6 hr. later. Total distance from fed blade to top of roots was 258 cm. Hence, minimum rate of translocation from leaf to root is 43 cm./hr. or 0.7 cm./min. A new estimate of speed of translocation was obtained when radioactivity was detected in roots 2 hr. after feeding a leaf. Total distance from fed blade to roots was 122 cm., giving a rate of 1.01 cm./min.

Climate has a striking effect on rates of translocation. In very recent experiments here (55) removal of new photosynthate from leaves fed C^{14} was slowed to about one-third the control rate by cooling roots 13°F. This emphasizes again the general circulatory system of which the roots are an integral part. Control conditions were: air temp., 81°F.; root temp., 73°F.; full sunlight. In each treatment one factor was changed: (*a*), half light; (*b*) air temp. 63°F.; (*c*) root temp. 60°F. The percentage of tagged photosynthate removed from the fed lamina the first 6 hr. (9 A.M. to 3 P.M.) was respectively: controls 82 per cent; half light 87 per cent; cold tops 61 per cent; cold roots 34 per cent.

Distribution of photosynthate is affected by nutrient status of the plant. A plant low in phosphate moved only 17 per cent of fed C^{14} to the growing point and young leaves compared with 44 per cent in the control plant. At the same time, the plant low in phosphate moved 57 per cent of fed C^{14} to

the stem below the fed leaf, compared with 37 per cent in the control. Thus the storage of new photosynthate in the stem is enhanced when use at the growing point is curtailed.

Translocation of C^{14}-2,4-D.—Sugarcane and bean differ strikingly in uptake and absorption of 2,4-D, according to studies by the authors [124 (p. 49), 126]. Radioactive 2,4-D painted on a bean leaf was rapidly translocated throughout the plant, even into roots. Not so with sugarcane, where most of the 2,4-D remained in the leaf where applied. Only one-tenth as much 2,4-D reached the growing point of the sugarcane plant as that of the bean plant, which may explain the high resistance of sugarcane to this chemical when applied in the field.

Flowering

The flowering process in sugarcane is extremely sensitive to the environment. This is true of flower initiation, tassel emergence, and pollen fertility. A variety which flowers heavily in one country may not flower at all in another at the same latitude and elevation. Even more striking responses to environment occur within a small area. Brett (164) gives an excellent discussion of some of the environmental effects.

Photoperiod.—Sartoris (165) and Allard (166) classified the variety of sugarcane with which they worked as a rare intermediate type, i.e., neither a long-day nor short-day plant but one requiring a day length between 11 and 14 hr. Burr (167) in 1946 prevented the flowering of variety H32-8560 by interrupting the night with incandescent lights. Thus sugarcane may be reclassified from Allard's intermediate class to a short-day plant which needs a long period of sunlight. The optimum photoperiod appears to be about 12½ hr. and most commercial varieties respond to this throughout the world. With very few exceptions flower initiation of commercial varieties occurs only in the fall days, i.e., six months apart in the northern and southern latitudes. The explanation that lack of flowering in the spring is due to low temperature seems more plausible than that shortening days are required.

Flowering occurs the year round on the equator [Hansford (168)] where daylight is almost constantly 12 hr. and 7 min. (excluding twilight). As distance from the equator increases the date of flower induction becomes more definite due to greater annual changes of day length. At Coimbatore, India (Lat. 11°N) flower primordia of the four varieties studied by Vijayasaradhy & Narasimhan (169) appeared at about the same time near the end of August. The 12½ hr. day occurs there about August 9th. In Hawaii (Lat. 19–22° N) the 12½ hr. day occurs about September 2 and lighting experiments indicate this is the approximate date on which initiation begins in this Territory [125 (p. 8), 167]. For all commercial varieties studied thus far initiation is complete by September 20, flower buds become visible under a hand lens between September 20 and 24, and no new buds appear after October 4 (25, p. 44–46). Gondo & Yoshimura (170) report September 23

to 25 as the date on which flower embryos of POJ-2725 become visible year after year in Taiwan (Lat. 23° N), irrespective of date of planting, soil type or exposure.

Both in India (169) and Hawaii (25, p. 44–46) it has been shown that time of appearance of flowers bears little relation to time of initiation. So-called late and early varieties may actually initiate primordia on the same date and differ by months in time of flowering, which depends upon the entirely separate process of elongation of the flower stalk. Within a single variety (H37-1933) the time of emergence of flowers extends from November to April, although it is demonstrated that the randomly selected controls all initiated in September. Some flowers fail to emerge at all and remain wrapped in the old sheaths. These "false tassels" can be produced in quantity by applying strong night light after September 20. This is too late to stop initiation but stops the growth of the flower stalk (88, p. 11–12).

Frequently embryonic flowers revert to the vegetative state, with the racemes producing leaves in a "bunch top" (171). One of these eventually takes over the continuing production of normal stalk joints (55). False tassels and bunch top are exceptionally common in young cane which has barely reached physiological maturity (puberty) in time for September floral initiation.

The highly reproducible short period of floral initiation makes possible the suppression of flowering in young cane on a commercial scale. It has long been thought that flowering reduced sugar yields [Hes (172)] but positive experimental proof could be obtained only after methods of suppressing flowering were available. Many replicated experiments in Hawaii have established that flower inhibition by night lights, chemical sprays or withdrawal of water can be expected to increase yields by 10 to 20 per cent under conditions of heavy flowering.

Attempts to induce flowers in nonflowering varieties or to change the date of flowering by applying artificial photoperiods have been on the whole unsuccessful (169). The only definitely successful inductions have been with varieties which flower readily by applying the natural favorable day length of about 12½ hr. (165, 173 to 176).

Temperature.—Small changes in temperature have large effects on flowering and pollen fertility. Brett (164) finds in Natal that flowering is maximum in a middle altitude belt, being less at sea level and ceasing entirely in cane fields at the highest altitudes. He also considers temperature the sole factor affecting pollen fertility. In Hawaii flowering is rare at 2000 ft. In controlled temperature houses it was shown that changing night temperatures ±5°C. inhibited flowering (53, p. 48–49). In Mauritius (177) open anthers decrease from about 90 per cent at sea level to none at 1200 ft. altitude.

Soil quality.—In some areas of Hawaii whole fields of luxuriant cane will have no flowers while on nearby alluvial fans flowering is heavy. That soil quality is at least one contributing factor has been demonstrated by Coleman (178) who raised cane plants in several soils in pots at several locations.

XUM

The lack of flowering is not due to poor growth and no definite chemical differences have yet been found to account for the soil effect.

Moisture.—Moist cloudy weather is known to favor flowering of sugarcane. Flowering is regularly less in the hot, dry, irrigated areas. Recent work in Hawaii [25 (p. 44–45), 54 (p. 50–51), 124 (p. 42–43)] has clarified some disputed points and it is now known that if irrigation is stopped in August in time to reduce moisture in the plant to a proper level in mid-September flowering will be prevented completely with no permanent injury to the plant. The low tissue moisture reduces the length of one internode to about half normal, but this loss is regained by greater than normal growth of the new joints after irrigation is resumed. Flower control by withholding irrigation has become the major commercial method.

Chemical sprays.—There are a number of chemicals which reduce tasseling. Among the most effective are 3-(*p*-chlorophenyl)-1,1-dimethylurea (CMU), Versene, maleic hydrazide, pentachlorphenol, and alpha naphthaleneacetic acid (ANA). Corrosive chemicals which burn a large area of leaf produce the same effect as mechanical defoliation which can completely stop flowering if done at the right time.

No chemical has been found which will induce flowers in reluctant varieties. Triiodobenzoic acid and coumarin while not inducing flower buds, do promote lateral bud development which is an indication of an auxin change (125, p. 8).

Nitrogen.—Heavy applications of nitrogen appear to reduce flowering. This effect is most noticeable in young cane which is expected to just attain physiological maturity (puberty) in time for the September initiation period. A heavy application of nitrogen may prevent any flowers and this may be attributed to physiological immaturity associated with succulence. Older cane in very high nitrogen culture solutions may flower heavily.

Other fertilizer effects are very variable and it is known that sugarcane flowers well in extremes of nutritional states.

Ripeness to flower.—In Hawaii sugarcane planted after June 1 or ratooned after July 1 will not be mature enough to flower heavily. Usually it is necessary for a stalk to have two or three mature basal joints to flower. In a young stool with stalks of many sizes only one or two of the largest may flower. Therefore, the term "percentage of flowering stalks" is really an expression of the number which have reached puberty at the critical date of initiation.

Climate and Growth

Of the three major climatic factors, temperature, light and water supply, the first two are almost inseparable in most field observations and will be treated here as a unit. Photoperiod has been included under Flowering and this discussion will be limited to light quantity.

Temperature and light.—Sugarcane is definitely a warm-weather, sun-loving plant. It thrives best and produces more sugar per month in hot,

sunny locations. At higher altitudes cropping time is lengthened. In the hot, bright regions of Hawaii crops reach their optimum maturity and yield in two years or less. At altitudes of 3000 ft. maturity is delayed a year or more. Sugar per month is reduced but the final yield per acre may reach 20 tons. The altitude limit for commercial cane in Hawaii is about 3000 ft. but near the equator it rises to 5000 ft., where, with brilliant, hot days (above 90°F.) and cool nights (50 to 59°F.), they succeed in raising 120 tons of cane per acre in two years. Barnes (179) states that the average crop age in East Africa increases seven months in rising from sea level to 4500 ft.

Stender (180) shows a close relationship of stalk length and diameter to temperature. In the month of least growth (February, mean temperature 70°F.) the increase in length was 0.53 ft. and in volume, 10.63 in.^{3} The following July (mean temperature 77.8°F.) gave increases in length and volume of 2.14 ft. and 31.12 in.^{3} respectively. Although there is an age bias in these figures it can be conservatively stated that winter growth of the major stalks was reduced to one-third the summer growth. Cornelison (63) demonstrated large varietal differences in growth response to season and to age. Das (181, 182) and Halais (183) have shown an extremely high correlation ($+0.951 \pm 0.012$) between elongation and the number of day-degrees adjusted for moisture. Such a high correlation would indicate that under the conditions of the measurements temperature was the chief growth-controlling factor. Evans (184) found an almost linear response to temperatures from 62.6°F. to 73.4°F., with no indication of a falling off. Length increased 3.56 times as fast at the higher temperature. Length and dry weight curves did not follow each other closely. Clements (185) in analyzing growth differences between two locations in Hawaii concludes that the marked differences in yield are due to sunlight rather than temperature effects. He and coworkers (186) find evidence that maximum daily temperatures of over 90°F. may be harmful. It seems to us that this may be a water effect, since higher temperatures do not cause signs of injury to canes in water culture (124, p. 41–43). Very large containers of well mixed soil were placed at Makiki (high sunshine) and Manoa (low sunshine) to eliminate possible soil effects on climate studies [H.S.P.A. (88)]. In two successive one-year crops it was found that efficiency of utilization of heat and sunshine for both cane and sugar production increased at the higher intensities, a rather surprising result. Relative amounts of sugar per 1000 day-degrees were 100 at Makiki and only 42 at Manoa. Doubling the hours of sunshine also more than doubled the crop. Experiments with sugarcane growing in culture solution in controlled temperatures and light have been going continuously at this Station since 1948 (88, 124, 125). Root and air temperatures are separately controlled. Four root and three air temperatures at two light intensities produce 24 climates running simultaneously in four replications. The results are too numerous and detailed to enumerate here but the following points will illustrate effects found.

(1) Below 70°F. root temperatures become strongly limiting to growth.

XUM

No growth takes place at 50°F. At 62°F. reducing sugars in the top joints fall to 5 per cent of the controls even though the tops are warm [Brodie (186a)].

An 80°F. root temperature is optimum for both growth and nutrient absorption. Dropping root temperature from 74° to 66°F. cuts P intake to one-third and reduces N intake to about one-half.

(2) When growth is controlled by root temperature water consumption and growth run parallel, resulting in a constant quantity of water used per unit of dry matter.

(3) Nitrogen content of leaves may be brought to every possible level by changing the ratio of root to air temperature. High-air, low-root temperatures result in very low leaf nitrogen for example. Other striking changes in composition occur and juice quality is affected.

(4) Cutting full Hawaiian sunshine in half reduces growth to about half.

(5) The effects of night temperature were shown by an experiment in in which sugarcane was grown under identical day conditions but at night one group was cooled to 57°F. and the other warmed to 73°F. After 20 weeks the cool nights reduced growth to approximately half that of the warm group. Weight of leaves and overnight translocation of sucrose from leaves to stalk were reduced to half.

For the satisfactory germination of the seed piece a soil temperature in excess of 70°F. is required (187). For most, if not all varieties, a soil temperature below 70°F. results in either very slow germination or complete failure. The optimum temperature lies between 90° and 100°F. Temperatures above 100°F. may prove detrimental (188). These effects will vary somewhat with variety.

The sensitivity of the seed piece to temperature is illustrated by the fact that a drop in soil temperature from 78° to 71.5°F. adds ten days to germination time of variety H32-8560 (182).

Other factors affecting the results of germination studies are size of cane used for the seed piece, number of eyes, and previous feeding of the seed plot. In plantation practice pieces with three or more eyes are used to minimize the chance of fungus infection. Treatment with hot water or with hot PMA (phenylmercuricacetate) at 52°C. for 20 min. protects the cane from disease and increases the rate of germination. Brandes & Overbeek (189) found that the hot water treatment reduced the apparent free auxin by as much as 50 per cent and lessened the dominance of the upper buds over the lower ones.

The rate of early growth of single eyes depends upon the quantity of stalk attached to them (88, p. 11). For this reason when an especially vigorous early growth is desired for an experimental study, two eyes of a three-eye seed piece are killed leaving only the central bud.

Water.—Evans (190) reported varietal differences in the amount of water lost by the sugarcane plant. However, the major factor which determines the amount of water loss is the environment, especially light intensity, air

humidity, soil temperature, air temperature, soil moisture, and wind velocity. In very detailed studies Lyon & Brodie (191) reported that even slight differences in light intensity, caused by drifting clouds, affect water loss. Duncan & Cooke (192) found that decreasing the soil temperature in the range of 28°C. to 10°C. decreased water loss.

Kuijper (193) considered light, temperature, humidity, and soil moisture as the dominant factors in stomatal movements. He found that the stomata were wide open in direct sunlight, partly closed in weak diffused light and first closed then slightly open in complete darkness. High humidity, high temperature, and irrigation promoted stomatal opening, and temperatures above 40°C. caused opening of the stomata in the dark. Evans (194) found no relationship between stomatal density and transpiration. Although there were twice as many stomata on the lower surface of a leaf as on the upper surface the transpiration rate was approximately the same from both surfaces. He concluded that the large bulliform cells with thin cuticle on the upper surface were largely responsible. The wax which covers all of the joint except the nodal region reduces the loss of water. Lauritzen (195) showed that dewaxed stalks have almost twice the transpiration rate of untreated stalks.

Several workers have determined the amount of water used by the sugarcane plant to produce a gram of dry substance. This ranges from 148 gm. [Maxwell (196)] to 300 gm. [Tempany (197)]. This large range is probably due to differences in variety, age, climatic conditions, and experimental techniques. By manipulations of root and air temperature and light intensity in the controlled glass houses at this Station great changes in efficiency of water have been effected. Values range from 129 ml. to 299 ml. of H_2O per gm. dry matter produced (55).

The absorption of water vapor by leaves was reported by Wadsworth & Das (198). Shaw & Wadsworth (199, 200) considered that dew and light showers contributed materially to the water supply of sugarcane. They reported that the sugarcane plant is able to absorb water through its aerial parts and discharge it through its roots into the soil. A plant of sugarcane growing in a steel tank, with the soil surface made water tight with an asphalt seal, gained 80 lb. in fresh weight after being sprinkled with a garden hose for 3 hr. There was no question about the seal being water tight since the moisture content of the top 6 in. of soil was practically the same after sprinkling as before, but the moisture content of the soil at depths below 6 in. was considerably increased after sprinkling.

The root pressure of sugarcane stumps may be as great as 140 cm. of mercury, [Weller (201)]. Root pressure showed diurnal variations with a maximum at night and a minimum during the day and was in direct proportion to the water content of the soil. Evans (106) found that a liquid was exuded from cut roots when left in the soil and positive pressures were obtained almost throughout the year if the soil moisture conditions were favorable.

XUM

Clements & Kubota (202) made an extensive study of the moisture content of 25 anatomical parts of the sugarcane plant by monthly samples from 2 to 22 months of age. In general the moisture content of a given tissue becomes less as the plant grows older. This is most extreme in the lower internodes and sheaths, and least extreme in the apical meristem and upper internodes. Other factors which affect the moisture content of tissues are variety [Cornelison (63)]; nutrients [Borden (203)]; light [Borden (203)]; and time of day [van Dillewijn (204), Hartt (143, 162), Wadsworth (205)].

The highest yields of sugarcane are realized on irrigated lands. About a ton of water is required to produce a pound of sugar on some irrigated plantations of Hawaii, [Baver (206)].

The major problem in irrigation is one of timing, that is, when to irrigate in order to obtain maximum yields at an economical level. A number of approaches to timing irrigations have been based on growth rates, soil moistures, tensiometers, Bouyoucos blocks, and meteorological data. The most widely used methods, other than the appearance of the crop, are soil moistures and the Bouyoucos block method introduced by Ewart (207).

Das (208, 209) used his concept of "day-degree" to arrive at the consumptive use of water and thereby determine the irrigation interval from meteorological data. He defined the "day-degree" as the number of degrees Fahrenheit between the daily maximum temperature and 70°F. About 350 day-degrees between irrigations appeared to be optimum. Swezey (210) found no consistent number of day-degrees accumulated between irrigation and the point of soil moisture exhaustion. The major weakness of the day-degree is that it is based on a single maximum temperature reading rather than an integrated temperature.

Fuhriman & Smith (211) reported that the consumptive use of water by sugarcane in Puerto Rico was 0.10 in. to 0.12 in. of water per day during the first and last part of the season and 0.18 in. during the season of peak growth. When the consumptive use of water data were used in the Blaney & Criddle (212) formula to determine the consumptive use coefficient (K) they found 0.86 for September, 0.57 for the preceding April, and 0.54 for the ripening period.

Cowan & Innes (213) studied the consumptive use of water and evaporation from a free water surface in Jamaica. After the sugarcane had established a complete canopy they found an evapotranspiration/evaporation ratio of 0.57 for lysimeter studies and 0.58 for field studies. They concluded that seasonal variation was small. Their data fit well into the Penman-Schofield equation. [See Cowan & Innes (213)].

In a recent study at this Station (55), moisture tension was increased daily by adding sucrose or NaCl to culture solutions. Measurements of spindle growth, sheath growth, and photosynthesis were made. Strikingly, growth of both tissues had ceased by the fifth day at 2 atm. pressure, while photosynthesis was still 75 per cent of normal.

In the same study it was shown that each increment of osmotic pressure reduced germination rate, which was inhibited entirely at 7 atm.

Fibrovascular water of Variety Q.50 has been recently estimated as 0.8 per cent of the total stalk volume by forcing KCl solutions through sections of stalk (214). This value agrees well with the estimate of vessel size made by microscopic examination of cross sections of the internodes. Expressed as proportion of the total cross section values given are: fibrovascular bundles, 5.3 per cent; vessels+sieve tubes+adjacent stained tissue, 3.1 per cent; vessels only, 0.8 per cent.

Ripening.—Moisture percentage drops steadily throughout the life of the sugarcane plant, from about 83 per cent in very young cane to 71 per cent in mature cane. Meanwhile sucrose rises steadily from less than 10 per cent to more than 45 per cent of the dry weight. Sucrose and moisture curves are almost exact mirror images of each other [Borden (77)]. Meanwhile nitrogen content drops along with moisture, a relationship so close that Samuels, Capo & Bangdiwala (215) found that a correction for moisture eliminated most of the fluctuation in leaf total nitrogen. Reducing sugars almost vanish from fully matured joints.

The above is the general picture of maturing cane, resulting in juice with a Brix of 20 or more, of which over 90 per cent may be sucrose. On irrigated plantations, where water can be controlled, it is a common practice to withhold water for a considerable period before harvest. This "drying off" hastens the above processes. As shown by Hartt (135, 136, 137) and Ashton (138) photosynthesis continues at a reduced rate after the wilting coefficient has been reached. In recent work at this Station (55) it has been shown that the sucrose-to-fiber ratio does indeed increase markedly with drying. This could result partly from conversion of reducing sugars to sucrose and partly from continued photosynthesis after growth is stopped.

Cornelison (63) found that the concentration of juice from the node differs greatly from that of the middle of the internode. Sucrose distribution along the entire stalk length as well as within single joints is discussed in great detail by Dillewijn (204). This is important in testing a field for maturity.

When pieces of sugarcane are pressed the first juice flowing from the easily ruptured pith storage cells may have a Brix of over 24. The value falls rapidly with increasing pressure and may drop below 14 for the final increment expressed at 5000 p.s.i. [Atherton (216)]. Ramiah & Varahalu (217) presented curves for Brix in individual joints superimposed on entire stalks, showing highest Brix in nodes of immature joints but in internodes of mature joints. Venkataraman & Row (218) analyzed that part of stalk bearing only dead leaves and the highest sucrose reading obtained was called the "sucrose index" of that variety. Opinion differed at first as to whether active storage of sucrose continues in the "dead leaf cane." Das (219) considered the storage of sugar in a joint nearly complete two to three months after it is formed, but Yamasaki & Arikado (132) proved the continued storage of sucrose in joints below the lowest green leaf, in studies of defoliation, which permanently decreased both Brix and sucrose not only of the defoliated section but also of all the lower internodes. Analytical evidence for the con-

XUM

tinued storage of sucrose in the dry-leaf cane was also presented by Clements (83) and by Hartt (137). Definite proof of storage in even the oldest joints has been obtained in our studies with C^{14}, reported by the H.S.P.A. (124, p. 55–56). Radioactive material formed from $C^{14}O_2$ fed to a single leaf of one stalk was found in sister stalks the following day. Harvested after eight days, greatest amount of the material had gone to the stalk immediately below the joint to which the fed blade was attached, but radioactive material was found even in the oldest joints.

The importance of improving the sucrose content of sugarcane rather than cane tonnage alone was emphasized by workers in Puerto Rico, who have presented an important series of papers on the effects of fertilizers [Samuels *et al.* (220)], varieties [Lugo-López & Capó (221)], soil conditions [Lugo-López (222)], and weather and climate [Lugo-López & Capó (223)].

Probably more work has been done on the effect of N fertilization on sugarcane than any other single factor, most workers finding, as reported by Borden (122, 224, 225, 226) that increased applications of N increase the yield of cane, decrease the percentage of sucrose, increase the percentage of reducing sugars and moisture, giving as over-all result more sucrose per acre. So it is with keen interest that we read the work of Samuels *et al.* (80), who studied more than 150 field experiments in Puerto Rico and stated that N increased both sucrose content and cane yield, a significant increase in sucrose per cent occurring when the increase in cane tonnage is over 27 per cent.

Many experiments in several sugar-producing areas have indicated that fertilization with K may increase the sucrose content. Samuels *et al.* (220) summarized 146 field experiments and concluded that when K fertilizers produced cane tonnage increases of 10 per cent or more, the sucrose content also invariably increased.

Findings differ as to the effect of P fertilization upon sucrose content. Most reports have indicated no significant effects [Geerts (227); McKaig & Hurst (228); Samuels *et al.* (220); Carey & Robinson (229)], but Cross (230) reported a negative effect, whereas Clements (231) and Feillafé (232) reported positive effects.

Water supply simultaneously affects growth, photosynthesis, translocation, storage, and juice quality to different degrees and the last word has not been said on how to balance these effects for maximum yield of sucrose "in the bag." In a study of climate, Hartt (233) found a positive correlation between moisture and reducing sugars and a negative correlation with sucrose; and in three studies of water and cane ripening (135, 136, 137) reported that a plentiful supply of water is essential for formation of sucrose in blades, its transport to the stem, and expression in the juice. Clements (8) recommended that for maximum production of sugar, sugarcane should be harvested when the sheath moisture falls to 73 per cent. Parthasarathy & Rao (234) stated that all factors affecting ripening do so by their effect on water content, and increased water content decreased sucrose content.

Shaw (235) found interesting trends in sucrose depending upon latitude, best sucrose per cent cane being obtained at 18° N and 18° S Latitude, with drops toward the Equator and rapid drops to 30° N and S, the apparent ecological limit for sugarcane. Causal agent suggested for this phenomenon was length of day.

Findings differ as to the effects of hormone sprays upon the sucrose content of sugarcane. Beauchamp (236, 237) obtained gains in sucrose following sprays with acetylene or 2,4-D, with some differences due to time of spraying, variety, and moisture conditions. His results were confirmed by Mathur (238) in India and by workers in Brazil (Anon. 239) but not by Loustalot *et al.* (240), Lugo-López & Grant (241), or Lugo-López *et al.* (242) in Puerto Rico.

Post-harvest deterioration of sugarcane.—In many of the sugarcane areas of the world climatic conditions and methods of harvesting frequently cause a delay between the cutting and milling of the stalks. During this period several types of deterioration take place. The stalks lose sucrose through the enzymatic process of inversion. The extent of inversion losses has been studied by Lee (243), Rosenfeld (244) and Sanyal (245). Lauritzen (246, 247, 248) working in Louisiana has investigated this problem quite thoroughly and reviewed most of the literature on the subject prior to 1948. Since 1948 there have been a few reports (249, 250, 251) of the extent of inversion losses under various conditions but no reports of work done under controlled conditions similar to that reported by Lauritzen.

Moisture loss appears to be the factor most closely associated with inversion of sucrose, and under storage conditions which minimize moisture loss the reduction of sucrose is likewise minimized. Some workers (243, 244) believed that high temperature was an accelerating factor in inversion losses but Lauritzen showed that between the temperatures of 45° and 75°F. moisture loss was a much more important factor. It is quite generally agreed that increased maturity of the sugarcane seems to increase the resistance to inversion of sucrose.

From parent and progeny studies of Lauritzen (252) it was concluded that certain degrees of susceptibility or resistance to inversion losses were hereditary among the different Saccharum species. However, among the varieties no correlation was found between invertase content and the rate of inversion.

Another source of sugar loss in the harvest of sugarcane stalks is that due to respiration, the extent of which has been reported by Lal & Srivastava (160), Hes (157) and Coleman (253). The sugar loss due to respiration in harvested cane can amount to a pound of sugar per ton of cane per day.

In sugar areas such as Louisiana, Florida, South Queensland and northern India additional harvest losses are frequently experienced due to frost injury. Most of the studies of this type of injury have been reported by Lauritzen and his co-workers (254, 255). There may be several degrees of frost injury ranging from leaf damage to damage to the terminal buds, lateral

XUM

buds and to the stalks of mill cane. Injury to the stalk destroys its use for the manufacture of sugar. In the field the deterioration of frost-damaged mill cane under warm humid climatic conditions is principally that due to the activity of micro-organisms which invade the damaged tissue. Such activity is accompanied by an increase in acidity which in turn accelerates inversion losses.

Burning cane in the field to remove trash introduces additional deterioration in sugarcane which is not promptly milled. With burned cane the deterioration due to the natural process of inversion is somewhat delayed due to the temperature effect upon the enzymes. There also exists under moist conditions deterioration due to the action of micro-organisms similar to that encountered with frozen cane. This subject has been adequately reviewed by Balch *et al.* (256).

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MASS CULTURE OF ALGAE[1]

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Introduction

During the past several years considerable attention has been given to the economic possibility of large scale mass culture of algae; there has been a great deal of speculation, discussion and experiment on the subject (1 to 7). The relevant work published before 1952 has been comprehensively surveyed in a monograph (8) compiled by the Carnegie Institution of Washington. In dealing with the recent advances in the study of algal mass culture, we may distinguish three different lines of development, namely: (*a*) the culturing of algae aimed at producing useful organic substances (food, feed, or some special organic material) as was originally suggested by Harder & von Witsch (9) and by Spoehr & Milner (10); (*b*) the culturing of nitrogen-fixing algae with the purpose of utilizing them to increase soil fertility; and (*c*) growing algae in sewage in symbiosis with bacteria with the dual purpose of accelerating the stabilization of organic sewage material and subsequently harvesting algae for the same purpose as (*a*). Not only the culture technique, but also the species of algae to be used are more or less different in each of these three lines of research. In (*a*) the most frequently used organisms are fast-growing green algae such as *Chlorella* and *Scenedesmus*; in (*b*), blue-green algae having strong nitrogen-fixing activity such as *Anabaena* and *Tolypothrix;* and in (*c*), mixed cultures of various algal strains which grow well in harmony with aerobic sewage bacteria. For convenience of reference we may call (*a*) mass culture of green algae, (*b*) mass culture of nitrogen-fixing algae, and (*c*) algal culture in sewage. Although different in their aims, these projects have many common basic problems relating to the physiology and biochemistry of algae which must be studied under well-controlled laboratory conditions. A characteristic of the physiology of algae is the variety and flexibility of their nutrient requirements as well as of the chemical composition of their cells. Earlier work on the growth physiology and metabolism of algae was reviewed by Brunel *et al.* (11), Blinks (12), Myers (13) and Fogg (14), and the nutrition of algae has been recently reviewed by Ketchum (15) & Pirson (16). Discussion of these problems is, therefore, largely omitted in this review, although they are of fundamental importance in relation to our subject.

Cultures under Controlled Laboratory Conditions

The necessary conditions for achieving vigorous algal growth are: (*a*) a supply of carbon dioxide and mineral nutrients in adequate concentrations,

[1] The survey of literature pertaining to this review was concluded in June, 1956.

XUM

(*b*) illumination with light of suitable intensity, (*c*) maintenance of favorable temperature, and (*d*) adequate agitation to prevent precipitation of the algal cells and at the same time to ensure even distribution of carbon dioxide, nutrients, and light.

Culture techniques.—Various techniques of indoor culture have been used to fulfill the above-mentioned requisites. There are four methods of agitation. (*a*) In the bubbling culture both the supply of CO_2 and agitation are effected by bubbling CO_2-enriched air through the culture. Culture chambers used include test tubes (17), various types of flasks or bottles (18 to 26), glass cylinders (27 to 30), annular glass tubes (29, 31), etc. (*b*) In the stirring culture agitation is obtained by using a stirrer driven by a motor or an air turbine. Culture chambers used are bottles or carboys (32), vats (33), tanks (34, 35), a lollipop-shaped chamber (36), or an annular chamber having a rotating stirring cylinder at the center [turbulent culture of Davis *et al.* (37)]. (*c*) The shaking or rolling culture is agitated by mechanical moving of the whole culture chamber. Containers are flat flasks or bottles (38, 39), Warburg vessels (40), or a bottle (laid on the side) with a rolling device (41). (*d*) In the circulation culture agitation is achieved by circulating the culture solution with a pump or a paddle-wheel. Containers used for this type of culture are plastic or glass tubes (25, 37), trays (42), etc.

Illumination is furnished either from the outside or by immersing fluorescent or neon tubes in the culture solution, the latter device usually being used in the case of bubbling cultures [e.g., Ketchum *et al.* (21)]. Except in the study of algal-bacterial symbiosis in sewage water, carbon dioxide is supplied by introducing CO_2-enriched air into the culture solution. The rate of gas supply and the concentration of CO_2 in the air vary according to the structure of the culture container and the degree of agitation of the culture. Nielsen (43) stated that when a very dilute algal population is used and the CO_2-containing air is supplied vigorously in small bubbles, the growth of *Chlorella* is independent of the CO_2 concentration down to 0.01 to 0.03 per cent. For ordinary experimental arrangements at least, a CO_2 concentration in the range of 1 to 10 per cent has been found to be adequate and is widely used in laboratory experiments (cf. 33, 37). Higher concentrations are thought to be toxic to algae, although Geoghegan (44) reported that no such effect was observed (with *C. vulgaris* var. *viridis*) when the CO_2 concentration was raised to as high as 20 per cent. According to Österlind (45), a certain strain of *Scenedesmus* (*S. quadricauda*), unlike *Chlorella*, utilizes bicarbonate better than undissociated CO_2 as the carbon source for growth. No attempt has been made, however, to grow this or any other strain of algae in mass using bicarbonate as the sole carbon source.

For the inorganic requirements of various algae, especially the difference in this respect between green and blue-green algae, the reader may refer to reviews by other authors (13 to 16; see also 23, 46 to 49). With sewage cultures, heterotrophic, mixotrophic, and auxotrophic growth of the algae must be considered (13, 14, 16, 51, 52). Unfortunately, these aspects of algal meta-

bolism have not been sufficiently investigated in connection with algal culture in sewage. Determination of the suitable relative concentration of each nutrient substance is a matter of importance in obtaining active growth of algae. While some species grow only within a narrow range of salt concentrations (52, 53), green algae such as *Chlorella* and *Scenedesmus* can grow with the same vigor over a wide range (54), a fact which makes these algae suitable for large scale mass culture. Nevertheless, to obtain steady growth in long range cultures measures must be taken to keep the concentrations of nutrients as well as that of hydrogen ions at suitable levels. The simplest way to achieve this may be to resuspend the algal cells in fresh medium at certain intervals (25). More rational is the replenishment method adopted by Krauss (33, 56, 57), who added the elements to the solution at the same rate at which they were removed by the algae (*Scenedesmus*). The rate of removal of each element from the solution had been made clear by analyses of both nutrient media and algal cells. Different in principle are the steady-state culture techniques devised by Cook (28) and by Myers and co-workers (31, 36). These devices maintain a constant population in a container by means of a photoelectric cell circuit which activates a solenoid valve allowing the entry of new medium. When the cell concentration increases above a predetermined point, fresh medium is automatically added, and the amount of overflow during a given period can then be used as a measure of growth. In their studies of algal culture in sewage, Gotaas *et al.* used two continuous culture techniques. One is a simple bubbling culture using a vertical glass cylinder (30, 58, 59). A definite volume of culture, depending upon the "detention period" to be studied, was removed each day and was replaced by an equal volume of fresh sewage water. The other type of culture, using an apparatus called a "symbiocon" (60, 61), is a closed system in which algae and bacteria are maintained in symbiosis without supply of atmospheric air. Nutrient sewage is continuously added and an equivalent volume of the culture withdrawn at a constant rate.

The difficulty of furnishing an adequate supply of microelements to the algae has been largely eliminated by the use of chelating agents which make possible concentrations of the elements higher than can otherwise be tolerated by the algae. Since Myers *et al.* (29), following the suggestion of Hutner *et al.* (62), first used ethylenediamine tetraacetic acid (EDTA) with success for the culture of *Chlorella*, this chelating agent has been widely used for algal culture (8). This agent had been thought to be, by itself, nonmetabolizable (62), but recent investigations by Krauss (33) using labeled EDTA for the culture of *Scenedesmus obliquus* indicate that it (or its breakdown product) is absorbed, although in a small quantity, and probably metabolized by the algal cells. Tamiya *et al.* (25) reported that, for the culture of *Chlorella*, EDTA may be replaced by nitroso-R salt or nitrilotriacetic acid. Whether or not these chelating agents are metabolizable by algal cells remains to be seen.

Growth of algae under continuous illumination.—Most of the useful data

XUM

concerning the kinetics of algal growth have been obtained with optimum carbon dioxide and nutrient salt levels (mostly using *Chlorella* as the organism). Under such conditions the rate of algal growth is a function of temperature and the intensity of light available to each algal cell existing in the culture, the latter in turn being a function of the population density. The probability of mutual shading of algal cells as a function of population density, cell size, and thickness of the culture along the direction of a light beam has been formulated by Tamiya *et al.* (25). Experimentally it was observed (25, 29, 55) that (*a*) when the effect of mutual shading is negligibly small, the growth process occurs "exponentially," i.e., $dN/dt = E \times N$, or $d\log N/dt = E$, where N is the quantity of algae contained in the culture, and E a constant, and (*b*) when mutual shading is so strong that the light is totally absorbed by the cell suspension, the growth process becomes "linear," i.e., $dN/dt = L \times A$, where A is the area of illuminated surface of the culture and L a constant. Both E and L are functions of temperature and the intensity (I) of incident light. At very low light intensities, they are both linear functions of I and are independent of temperature (light-limited growth rates: E_o, L_o); at sufficiently high light intensities, E becomes independent of I (light-saturated: E_s), whereas L continues to increase, although the rate of increase gradually tapers off with the increase of light intensity. Unlike E_o and L_o, E_s and L at higher light intensities are distinctly temperature-dependent (25, 63). Assuming Beer's law to hold in the algal suspension, attempts to derive a formula relating L to I were made by Tamiya *et al.* (25) and later by Kok (64) and Oorschot (19). By expressing the I-dependence of E in the form of a simple rectangular hyperbola, Tamiya *et al.* obtained an L-I-relationship of logarithmic function (containing terms for E_s, E_o and the extinction coefficient of algal suspension) and the agreement between calculated and observed values was proved experimentally at different temperatures (63). On the other hand, Oorschot used an exponential function for the E-I-relationship, and by its integration came to a very complicated L-I-relationship in the form of an "Ei" function. Using *Chlorella ellipsoidea* as material, Tamiya *et al.* (26) made a quantitative analysis of the over-all process of algal growth based on the assumption that it is composed of two main phases, photosynthesis and light-independent formative metabolism. It was revealed that the light-independent phase has a remarkably greater temperature coefficient than photosynthesis. It was also shown that at 25°C. the light-saturated growth rate (E_s) is largely determined by the process of photosynthesis, whereas at 7°C. the light-independent phase represents the main rate-determining step in the over-all growth process.

The rates of exponential growth (E_s) shown by various algae have been listed by Myers (13, 55). The highest growth rate yet observed with an alga is that of a blue-green alga, *Anacystis nidulans* (50), whose E_s value (in terms of $\log_{10}$ units per day) was 4.2 at 41°C. This corresponds to a 16,000-fold increase of cell quantity per day (under continuous illumination). A relatively fast growing *Chlorella* (*C. ellipsoidea*) shows E_s values of 0.09 at 7°, 0.48 at

15°, and 1.2 at 25°C., which correspond to 1.2-, 3-, and 16-fold increases per day, respectively (25). The rate of growth (L) in the linear phase is usually expressed in terms of daily increase in grams (dry weight) of algae per square meter of illuminated surface. In most indoor experiments the algae are grown under continuous illumination, while outdoor cultures are subjected to a diurnal intermittency of illumination. For convenience of comparison, therefore, the data of indoor cultures may be expressed in terms of gm. per m.[2] per 12 hr. of illumination. As a measure of growth activity of algae we can also use the efficiency of light-energy conversion, which can be calculated by determining the heat content of algal cells formed,[2] and the quantity of photosynthesizable light absorbed.[3] The efficiency can be gauged both for exponential and linear phases of growth, but for technical reasons, it is more easily measured in the linear phase, where all the incident light is absorbed by the cell suspension. In Table I are reproduced some data obtained with *Chlorella* under different light intensities and at different temperatures. It may be seen from the table that, in the range of temperature and light intensity studied in these experiments, the higher the temperature and light intensity, the higher the linear growth rate. The highest value observed (at 25°C. and with light of 50 to 75 kilolux) was 32 to 43 gm./m.[2]/12 hr. which corresponds to a yield of 47 to 64 metric tons per acre per year. The efficiency of light-energy conversion also increases with temperature, but, unlike the rate of growth, it decreases with an increase in light intensity (19, 65, 66). Extensive studies made by Kok (40), using *C. vulgaris* (grown at 25°C. with yellow sodium light), showed that the maximum efficiency attained was 24 per cent, a figure which agrees well with the maximum efficiency given in the table. The efficiency was also measured by Aach (65) using an Osram-lamp HNG, which emitted light in the wavelength range from 400 to 700 mμ. In one experiment, in which the efficiency of light-energy conversion was 6 per cent, 9.2 gm. of algae (*C. pyrenoidosa*) were obtained per kilowatt-hour of electricity consumed. If 25 per cent conversion-efficiency can be attained with the same illumination system, we can expect a maximum yield of 38 gm. per kw.-hr., or 1 lb. of algae per 12 kw.-hr. of electricity. The table shows that the yield was exceptionally low at 7°C. when the light intensity was above 25 kilolux. This was caused by the bleaching of algal cells, which usually occurs when the algal cells are exposed to an excess of light at low temperatures (19, 25, 37, 69).

Attempts at accelerating algal growth by physical or chemical means.—The fact that the efficiency of light-energy conversion is lower at a stronger light intensity has led several investigators to devise methods of accelerating algal growth by "diluting," either spatially or temporally, the incident light. Davis *et al.* (37) have demonstrated that the linear growth rate of *Chlorella* is markedly—as much as 70 per cent—increased by vigorous stirring of the

[2] The heat content of *Chlorella* cells ranges from 5.5 (44) to 5.8 kcal./gm. (35, 65).

[3] The visible light in the wavelength range from about 370 mμ to 700 mμ.

XUM

TABLE I

LINEAR GROWTH RATE AND THE EFFICIENCY OF LIGHT-ENERGY CONVERSION OF *Chlorella* GROWN UNDER DIFFERENT LIGHT INTENSITIES AND AT DIFFERENT TEMPERATURES. (INDOOR CULTURES ILLUMINATED WITH ARTIFICIAL LIGHT.)

Algae	Method of culture	Light intensity (kilolux)	Temp (C.)	Rate of linear growth (gm./m.²/12 hr.)	Efficiency of light-energy conversion (%)*	References
C. pyrenoidosa	turbulent culture†	75	25°	43	7.1	(37)‡
	bubbling culture	75	25°	25	4.1	
C. "Strain A"	stirring culture	30	32°	9.7	4.7	(35)§
		4.1	24°	3.9	13.3	
		3.4	32°	2.6	10.9	
C. vulgaris var. *viridis*	bubbling culture	14	25°	7.7	20	(44)
C. ellipsoidea	bubbling culture	50	25°	32	7.9	(25, 63)‡
			15°	14	3.4	
			7°	(0)‖	(0)‖	
		25	25°	23	11.4	
			15°	11	5.4	
			7°	(1.0)‖	(0.5)‖	
		10	25°	14	17.2	
			15°	7.5	9.2	
			7°	2.5	3.1	
		5	25°	8.2	20.2	
			15°	6.5	16.0	
			7°	1.8	4.4	
		2	25°	3.9	24.0	
			15°	3.6	22.2	
			7°	1.3	8.0	

* Efficiency was calculated on the basis of visible light received by the culture.

† This experiment was performed by Davis *et al.* (37) to investigate the effect of turbulence of a culture upon the growth rate of algae. The culture chamber used was a lucite annulus having a fast rotating stirring cylinder at the center. The result given here was obtained when the rotor speed was 208 to 475 r.p.m.

‡ For these data, the efficiency was calculated on the basis of the assumption (*a*) that the energy content of algal cells is 5.5 kcal./gm., and (*b*) that 1 kilolux in the wavelength range of 400 to 700 mμ is equivalent to 0.0062 cal./cm.²/min.

According to the data given by Moon (67) [cited by Brooks (68)] we may assume that radiation in the wavelength range from 400 mμ to 700 mμ comprises 44.3 per cent of the total direct sunshine at sea-level (sun's altitude: 30°). On the other hand, according to the measurement made by the Central Meteorological Observatory, Tokyo, 1 kilolux corresponds to the total solar radiation of 0.0132 to 0.0147 (average 0.0140) cal./cm.²/min., the value varying according to the sun's altitude (from 10.2° to 70.0°). Taking the average value it is calculated that 1 kilolux corresponds to 0.0062 cal./cm.²/min. in the wavelength range of 400 to 700 mμ.

§ In this study (35) the incident light-energy was originally given in terms of kcal./m.²/day, which was calculated into kilolux on the basis of assumption (*b*) described above.

‖ For these exceptionally small values, which were caused by the bleaching of algal cells, see the text.

culture (see the data given in Table I). This is attributed to the effect of intermittent illumination of individual cells, an effect which may be interpreted as equivalent to "dilution" of strong flashing lights to weaker lights of longer duration. By chopping the light beam with rotating sectors, Kok (70) and Phillips *et al.* (71) investigated the effect of light-intermittency upon the growth of *Chlorella* as it changed with the duration of the flashes, dark intervals, and the intensity of flashing light. Based on his earlier studies (72) on the effect of flashing light upon the photosynthesis of *Chlorella*, Tamiya (25) concluded that the growth-promoting effect of light-intermittency becomes insignificant at lower temperatures and lower light intensities (and, of course, at lower population densities). The conclusion arrived at in all these studies is that the marked growth-promoting effect of light intermittency can only be achieved when the cell suspension is agitated with enormous vigor, a condition which might be hardly feasible economically in large scale algal cultures. Another attempt at "diluting" light was made by Sasa (73) who suspended in the culture small particles of a phosphorescent substance (copper-activated Zn-Cd-S-phosphor) which emitted light at 560 to 570 mμ with a half life of 2 to 3 min. Experiments showed, however, that neither the rates of photosynthesis nor growth (in the linear phase) were increased in the presence of this substance in any concentration.

Some authors (74, 75, 76) have reported that the growth of *C. vulgaris* is stimulated by indoleacetic acid or related substances in suitable concentrations. Experiments performed by Leonian *et al.* (77, 78), however, gave distinctly negative results, showing that the reports made by earlier workers were based on some experimental errors. Thus far, no growth-promoting effect of auxin-like substances has been observed with certainty with *Chlorella* or *Scenedesmus*.

Synchronous culture.—All the investigations described above have dealt with the growth in bulk of algal populations without going into the details of physiological and biochemical changes occurring in individual cells in their life cycles. Studies on the latter problem in *Chlorella* became possible by the device of "synchronous culture," in which practically all the algal cells existing in the culture grow and divide almost simultaneously (26, 79). Various new facts revealed by using this culture technique can be only briefly reviewed here, although they have, in many respects, a fundamental bearing on the problem of the mass culture of algae. In their earlier studies, Tamiya *et al.* (80) distinguished two cell-forms, "dark cells" (D) and "light cells" (L), in the life cycle of *Chlorella*. The former are smaller in size, richer in chlorophyll, stronger in photosynthetic activity, but weaker in respiration than the latter. When illuminated, dark cells increase in mass and turn into light cells. The latter, when ripened, bear a number—2, 4 or 8—of autospores which then become individual dark cells. The division of light cells into dark cells can proceed only under aerobic conditions, but independently of light. Later studies (79) have revealed that both in dark and light cells there are different developmental stages which are distinguished as follows:

XUM

(*a*) "nascent dark cells" (D_n) which are the young cells newly produced from mother light cells; (*b*) "active dark cells" (D_a), the most chlorophyll-rich and photosynthetically active cells which are derived from D_n when illuminated; (*c*) unripened light cells (L_1), which are sufficiently large in size but not yet ripe enough to perform cell division; (*d*) half-ripened light cells (L_2); and (*e*) ripened light cells (L_3) which are ready to form autospores. The life cycle of algal cells proceeds in the sequence $D_n \rightarrow D_a \rightarrow L_1 \rightarrow L_2 \rightarrow L_3 \rightarrow n \times D_n$, where n signifies the number of daughter cells (D_n) produced from one mother cell. The activity of photosynthesis (under saturating light) as well as the quantum yield of photosynthesis fall off markedly at the stage of light cells (especially at L_2) [Nihei *et al.* (81); see also Emerson *et al.* (82)]. Nihei (83) found that when light cells at stage L_2 were illuminated in the presence of phosphate they consumed a considerable amount of phosphate with simultaneous evolution of oxygen. As a consequence, light cells at that stage show an apparent photosynthetic quotient (O_2/CO_2) high above unity (as high as 3) in the presence of phosphate, whereas they show a normal photosynthetic quotient in the absence of phosphate and in the presence of CO_2. The phosphorus fixed photochemically by the light cells was found, in the main, in an energy-rich form, probably in the form of metaphosphate. The occurrence of this substance in *Chlorella* cells has recently been reported by some other workers (84, 85, 86). Iwamura *et al.* (79, 87) have followed the fates of ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) during the cycle, and found that in light cells DNA is formed, probably at the expense of some part of RNA, in an amount which is closely proportional to the number of D_n formed from light cells.

Diurnal rhythm of growth and the effect of diurnal changes of light and temperature.—Under natural growth conditions there occur not only diurnally intermittent variations of light intensity, but also variations in length of day and night, accompanied by diurnal changes of temperature. Some algae, such as *Hydrodictyon reticulatum*, are so strongly adapted to diurnal intermittency of light that they cannot grow under continuous illumination (88 to 91). With this alga as well as with diatoms (92, 93), *Scenedesmus* (94), *Chlorella* (18, 65, 80) and *Ankistrodesmus* (88) it was observed that when the algae were subjected to diurnal changes of light and dark, cell division or changes in certain cell activities occurred periodically with the rhythm of light-and-dark alternation, and in most cases the periodicity thus evoked continued for sometime after a change from intermittent illumination to continuous light or darkness. Apparently, the growth of the bulk of the cell population in these cases had been made more or less "synchronous" by the effect of light-and-dark alternation, and the periodical change observed may be due to physiological differences of cells at different developmental stages in the sense of Tamiya *et al.* (26, 79).

Using *Chlorella* as the material, the effect of the change of day and night temperatures upon the rate of growth has been investigated by some workers (37, 66). Davis *et al.* (37) reported that the yields from cultures grown at

25°C. continuously were exceeded by using temperatures of 25°C. in the day and 15°C. at night. This was not corroborated in the experiments of Tamiya *et al.* (69) who investigated not only the effect of changing day and night temperatures, but also the effects of variations in day-length and daylight intensity upon the growth (exponential phase) of *C. ellipsoidea*. They found that the rate of algal growth was affected by changes of day temperature far more profoundly than by changes of night temperature. In the temperature range studied (7°, 15°, and 25°C.), the higher the day temperature, the higher the growth rate. Higher night temperatures had no recognizably favorable effect except when the day temperature was as low as 7°C. The effect of day-length was studied by using a 6-hr., 12-hr., and 18-hr. day, keeping the diurnal period always at 24 hr. In general, the growth rate was directly proportional to the day-length ("day-limited growth") at shorter day-lengths. Such a proportionality extended to longer day-lengths, the lower the daylight intensity or the higher the day temperature. At longer day-lengths, and especially under stronger daylight, the growth rate tended to become independent of day-length (the phenomenon of "day-saturation"[4]) or to become rather smaller with the increase of day-length (the phenomenon of "day-oversaturation"). Both day-saturation and -oversaturation occurred *ceteris paribus* more markedly at lower day temperatures. It was also observed that the temperature-dependence of the growth rate decreased and eventually tended to disappear with the decrease of daylight intensity and with the shortening of day-length.

Outdoor Mass Culture of Green Algae

Types of culture units.—To run outdoor algal mass cultures efficiently and economically, the population density of algal cells must be kept at a reasonably high level during the operation because it is necessary that the light falling on the surface of the culture be totally absorbed. In addition, a high density will reduce the cost of water handling in the process of separating the algal cells from the medium. Since at high population densities the increase of algal cells in the culture becomes proportional to the area of illuminated surface, culture units for outdoor mass cultures must be designed so as to have a large ratio of illuminated surfaces to volume. This condition has not necessarily been fufilled in the devices used in earlier studies. Attempts made in small-scale outdoor or semi-outdoor cultures (i.e., cultures run in green houses) were: (*a*) bubbling cultures, using large bottles or carboys (23, 24, 37), concrete tanks (97, 98), vertical or tilted glass tubes (29, 37), annular glass tubes (29), an upright flat chamber having transparent plastic walls (44), or a glass cylinder mounted on a pivot so that the cylinder may be tilted at different angles towards the sunlight (28); (*b*) stirring cul-

[4] The phenomenon of day-saturation under certain conditions has also been reported for *Chlorella vulgaris* var. *viridis* by Geoghegan (95) and for *Euglena gracilis* by Gotaas *et al.* (96).

XUM

tures, using large vats (24) or concrete tanks (35); (*c*) circulation cultures, using long plastic or glass tubes (25, 37) or a tray (42); and (*d*) shaking cultures, using a rocking tray (37). Besides these, several devices have been proposed for "diluting" sunlight, which, however, have not been tried experimentally. They are: (*a*) the use of surfaces inclined towards the mean position of the sun so that a thin layer of culture on the surface would be exposed to oblique illumination of lower intensity [Woods, cited by Burlew (8)]; (*b*) the use of vertical transparent tubes penetrating a deep culture and containing reflecting cones, base down, to reflect the light horizontally into the culture (99); and (*c*) the use of light-diffusing cones with their bases at the illuminated surface (100).

Outdoor cultures actually run on a rather large scale are of four types. (*a*) The "closed circulation system," in which the culture is circulated either in a plastic tube (101 to 104), or in a shallow concrete trench covered with plastic ceiling (105). (*b*) The "open bubbling system," in which the culture contained in an open shallow trench is constantly agitated by the aeration of CO_2-enriched air (97, 98, 104, 106, 107). (*c*) The "deep culture system," in which growth takes place, with constant stirring, in a deep concrete tank (1 m. in depth) having a transparent side wall directed to the south (108). (*d*) The "open circulation system" with a device for intermittent sweeping, in which the culture, in an open shallow pond, is circulated by means of movable pipes having many small holes for the ejection of culture liquid (104). The pipes are submerged in the culture solution and automatically rotated in the horizontal plane by the power of the water flux from the holes; this water flux simultaneously serves to agitate the culture intermittently.

Except in the case of the "deep culture" of Mayer *et al.* (108), the depth of the culture solution is in the range of 5 to 20 cm. The largest single culture unit thus far constructed is an "open circulation system" now operating in Tokyo, which has an illuminated surface of 78 m.^{2} and contains 7.8 metric tons of culture with a depth of 10 cm. Culture systems (*a*), (*b*) and (*d*) have been run in parallel by Tamiya and coworkers, and their advantages and disadvantages have been discussed in detail (104).

Selection of algae.—The selection of suitable algal strains is as important a matter as the design of the culture unit. Thus far, the search has been mostly for fast-growing, hardy, algae, although other features, such as the chemical composition and nutritive value, are also important. The choice of algal strains may influence the choice of culture conditions as much as the culture conditions influence the choice of the algae. Other conditions being equal, the desiderata for an algal strain are (*a*) that it be resistant to contamination by other organisms, (*b*) that the tendency toward frothing or excretion of organic matter from the cells during growth be as little as possible, and (*c*) that the cells be neither so large as to precipitate easily during culture nor so small as to cause difficulties in the process of separation from the culture medium. In most of the outdoor cultures strains of *Chlorella* and

Scenedesmus have been used because many of them fulfill, although not always satisfactorily, the above-mentioned conditions.

The discovery of a high-temperature strain (resistant to temperatures as high as 42°C.) of *Chlorella* ("Tx 71105") by Sorokin & Myers (109) has broadened our concept of the upper limit of culture temperature, which had previously been thought to be about 35°C. (cf. 65). A number of similar temperature-tolerant strains (15 *Chlorella* and 1 *Scenedesmus*) also have been isolated from soils and waters collected around Tokyo (104, 110). According to Kok (66), a special strain of *Scenedesmus* ("Strain 3") was found to be very flexible in its temperature characteristics, growing well not only at 20°, but also as high as 45°C. He also succeeded in adapting some strains of *Chlorella* to high temperatures. Attempts to adapt these strains to high light intensities proved unsuccessful, however. According to Gummert *et al.* (97, 98, 111), *S. obliquus* is far more resistant to contamination than *Chlorella pyrenoidosa*. The reported production of antibiotic or autotoxic substances in some algae (23, 112 to 116), does not seem to be a common characteristic of *Chlorella* and *Scenedesmus* [cf. Myers (13)]. The tendencies toward frothing and settling of cells differ widely even among strains of the same algal species (107). According to Meffert *et al.* (98), cells of *S. obliquus* precipitate more easily than those of *C. vulgaris*, while Krauss (33) states that *C. pyrenoidosa* has more tendency to settle than *S. obliquus*.

The idea of growing marine *Chlorella* in sea-water is an attractive one from a practical point of view. Loosanoff (117) grew a strain of marine *Chlorella* in a shallow wooden tank placed in a green house and tested its applicability as feed for mollusks. While most attention has been given to the culture of unicellular algae, Tamiya [cited by Moyse (118)] proposed the use of filamentous green algae, such as *Ulothrix* and *Hormidium*, as materials for mass culture. His reasoning was that (*a*) they grow on the surface of the culture solution (because of bubbles of photosynthetic oxygen produced by cells and held between the entangled filaments) so that they can be grown in relatively deep culture ponds, and, moreover, can be harvested very easily, and (*b*) they can be easily dehydrated by merely squeezing or pressing the algal mass. Culture experiments in the laboratory using *Ulothrix* sp. showed that its growth rate was comparable to that of a fast growing *Chlorella*, provided that the alga was in its "vegetative form." This form could be kept practically indefinitely when the culture was illuminated continuously at a constant temperature (25°C.). When transferred to an outdoor culture, however, this alga showed a periodic change of state, from vegetative to zoospore and back again; in the zoospore condition growth ceased almost completely and the culture was eventually eliminated and replaced by rapidly growing unicellular green algae. Several attempts were made to keep the alga in its vegetative form under outdoor conditions, but they proved utterly unsuccessful; thus, the idea of using filamentous algae as material for mass culture was tentatively abandoned.

XUM

As shown by this case, observations made in the laboratory cannot always be extrapolated to events occurring under field conditions, where environmental factors vary in a manner so irregular as to almost defy any attempt to duplicate them artificially. To obtain straightforward information about the growth characteristics of algae under natural outdoor conditions, Kok *et al.* (19, 66) ran a series of small-scale bubbling cultures using "Kolle" dishes which were held at an angle of 45° and exposed to the south. Similar experiments were performed by Sasa *et al.* (110) using oblong flat flasks; in this case the main body of the flask was dipped in shallow stagnant water, so that the temperature of the culture changed in a manner similar to that of large-scale open cultures. By running in parallel a series of large-scale open bubbling cultures it was found that, in each season of the year, these test cultures reproduced fairly well the growth characteristics of algae in large-scale cultures. The method adopted by Sasa *et al.* seems to be the most convenient and reliable one for selecting algal strains to be used in mass cultures.

Procedures and incidental measures taken in running the culture.—Although the procedures to be used in growing mass cultures vary according to the culture device and the algal strain used, there are some general problems which apply to all. The device for automatic control of culture conditions, such as used in continuous culture by Cook (28) and Myers *et al.* (31, 36), has not yet been attempted in large-scale outdoor cultures. In general, the population density and the composition of the culture medium are maintained only roughly constant by harvesting the algal cells occasionally and by replenishing the culture medium with the mineral nutrients consumed. For keeping the composition of the culture medium constant, the replenishment method proposed by Krauss (33), already mentioned in an earlier section, seems to be rational and most practical. The only problem is that there is a possibility of algal cells being subjected to a change in chemical composition according to the condition or the season of culture (22, 27, 65, 98, 119 to 123). To use Krauss' method satisfactorily we have to adjust the composition of the replenishment medium from time to time, taking into account possible changes in elementary composition of algal cells. In order to obtain a high yield of algae, aeration (O_2 supply) of the culture must be continued day and night (37), while the CO_2 supply may be stopped during the night. The provision of O_2 is necessary for cell division, as was shown by Tamiya *et al.* in their study of the life-cycle of *Chlorella* (26). When CO_2 is prepared by burning coal, it must be freed of sulfur dioxide and hydrogen sulfide before being introduced into the culture (98, 99).

An adequate initial inoculum and the maintenance of an adequate population density in a steady state (or quasi-steady state) are also important for a satisfactory yield of algae. The initial inoculum must be larger in winter than in warmer seasons. This is because the bleaching of cells caused by an excess of light occurs more easily at lower temperatures (37, 69). On the other hand, the steady state population density seems to be better kept lower in

winter than in summer. In any case, too high a density is undesirable because it reduces the chances for each cell to receive sunlight and consequently renders the algal population as a whole unhealthy. The highest population density ever recorded is 55 gm./l. (dry weight) which was attained by Myers *et al.* (29) in their outdoor culture of *Chlorella* using a glass annulus, in which the thickness of culture was 0.5 cm. Such a high density is hardly practicable in large-scale mass cultures in which the depth of culture is around 10 cm. or more. In the open circulation system used by the writer's group (104), the steady state population density is usually kept at approximately 1 to 2 gm./l. in winter and 2 to 4 gm./l. in summer, and the inoculum size is adjusted so as to give an initial population density of about 0.1 gm/l. in winter and 0.02 gm./l. in summer.

A serious trouble encountered in outdoor cultures is contamination by other microorganisms. Contrary to the general belief (cf. 124, 125) the closed culture system, as compared with the open system, has no special advantage in this respect, in as much as it cannot be run under strictly aseptic conditions (104). The contaminants thus far reported are various species of protozoa, rotifers, some bacteria, fungi and blue-green algae (37, 97, 98, 99, 101, 104, 106, 116), among which the protozoa and rotifers are most harmful. In the worst cases, they eat up the whole algal population within one or two days (97, 104). The trouble caused by these micro-animals can be eliminated successfully by the application of 2,4-dinitro-6-cyclohexyl phenylacetate or pentachlorophenyl acetate, which in very low concentrations (1 to 3 p.p.m.), kills the animals without affecting the growth of green algae (104).

In many strains of algae, marked frothing occurs during growth, especially when the population density becomes high (44, 101). This renders the algal yield small since the foam layer on the surface reduces the light to as little as $\frac{1}{3}$ to $\frac{1}{4}$ of the incident light intensity. The use of some commercial antifoam agents has been suggested (32, 44, 101), but judging from experience in the writer's institute, their effectiveness does not last longer than one day. Better means to circumvent the trouble of foaming may be to select strains having little or no tendency toward froth; such strains are not infrequent among species of *Chlorella* and *Scenedesmus*.

In connection with the project of mass culturing of green algae, there are in addition a number of technical problems such as the method of separating algal cells from the solution, the process of drying the cells, etc. Here, however, a detailed discussion of these subjects would be inopportune.

Yield of algae.—The highest outdoor growth rate recorded in the Carnegie Monograph (8) was 17.8 gm./m.^{2}/day [Davis *et al.* (37)], which was obtained in a closed bubbling culture of *Chlorella* (in a slightly tilted glass tube). This experiment was run in November when the culture received only 8 to 9 hr. of sunlight daily, but the temperature of the culture was artificially regulated to the most favorable values (30° day and 20° night). From this result Davis *et al.* inferred that in June and July with 12 to 15 hr. of sunlight a yield of about 30 gm./m.^{2}/day may be expected. The large-scale outdoor cultures

XUM

described in the Carnegie Monograph gave, however, rather discouraging results, the yield during the most favorable period of the year being only around 7 to 11 gm./m.²/day (35, 101). In the past few years both the culture techniques and the algal strains used have been improved considerably, and it is now possible to obtain a yield of as much as 20 to 28 gm./m.²/day on fair days in the warmer months. The improvements made in the past five years may clearly be seen from the figures given in Table II, in which the data reported by different authors are listed in chronological order.

TABLE II

AVERAGE AND MAXIMUM YIELDS OF ALGAE OBTAINED FROM VARIOUS TYPES OF OUTDOOR MASS CULTURES RUN DURING THE MOST FAVORABLE SEASON OF THE YEAR

Culture method	Algae	Month	Average (& maximum) yield in gm./m.²/day	Average efficiency of energy-conversion (%)*	Year of experiment (reference)
Stirring culture in a tank	*Chlorella* "Strain A"	May–October August–October	7.2 4.9	2.6 2.7	1951 (35)
Closed circulation culture	*Chlorella pyrenoidosa*	July–September	9 (11)		1951 (101)
Open bubbling culture	*Scenedesmus obliquus*	July August	5.7 6.6	— 2.7	1952 (98)
Stirring culture in a tank	*Chlorella* "Strain A"	May June August	10 (12) 11 (13) 8 (9)	3.7 5.4 4.5	1952 (19)†
Open bubbling culture	*Chlorella* "Cn 6"	May June	13 (20) 17 (28)	5.0 6.4	1954–55 (107)§
	Chlorella "Ctm 37"‡	August	16 (21)	7.1	
	Scenedesmus "Stm 32"‡	September	12 (16)	6.0	
Open circulation culture	*Chlorella* "Ctn 5"‡	July August	19 (24) 15 (19)	6.0 5.9	1955 (104)§
Stirring culture in a deep tank with a transparent side wall	*Chlorella vulgaris*	April May	19 21		1955 (108)‖
	Chlorella "Tx 71105"‡	June	20		

* The efficiency of light-energy conversion was calculated on the basis of visible radiation received.
† Figures given here are those estimated from the diagram presented by Oorschot (19).
‡ High-temperature strains.
§ The heat content of algal cells was assumed to be 5.5 kcal./gm. (cf. 44).
‖ In calculation of the yield, both top and side illuminated area were taken into consideration.

In this Table, it should be noticed, only yields obtained during the most favorable season are given for comparison. In other seasons, if the culture temperature is not artificially regulated, the yield is usually less, and in extreme cases even reduced to zero. The seasonal variation of growth rate, which is characteristic of each strain of algae, has been extensively studied by Sasa *et al.* (110) using the series of small-scale test cultures already described. Representative data obtained by them using mesophilic[5] and thermophilic strains of *Chlorella* are presented in Table III. As may be seen from the

TABLE III

SEASONAL VARIATION OF GROWTH RATE AND THE EFFICIENCY OF LIGHT-ENERGY CONVERSION SHOWN BY MESOPHILIC AND THERMOPHILIC ALGAE GROWN IN SMALL-SCALE TEST CULTURES*

Month	*Chlorella ellipsoidea* (mesophilic)		*Chlorella "Ctm 37"* (thermophilic)	
	Average growth rate in gm./m.²/day	Efficiency of energy conversion (%)†	Average growth rate in gm./m.²/day	Efficiency of energy conversion (%)†
January	4.4	2.9	0	0
February	6.3	3.8	0	0
March	12.3	6.0	1.0	0.5
April	17.6	8.6	5.1	2.5
May	17.5	8.0	6.6	3.1
June	15.5	9.6	6.5	4.1
July	12.8	6.9	10.3	5.5
August	7.2	3.2	12.1	5.3
September	12.2	6.0	12.5	6.1
October	16.5	11.9	12.3	8.8
November	16.3	12.0	3.8	2.8
December	6.1	5.2	0	0

* From Sasa *et al.* (110).

† Calculated on the basis of visible radiation received, which was assumed to comprise 44.3 per cent of the total radiation.

Table, a mesophilic strain, *C. ellipsoidea*, showed positive growth in all months throughout the year, although its growth was considerably suppressed in the hottest month, August. In contrast, the thermophilic strain grew actively in the summer months, but showed no growth in the winter months (December, January, and February). From these observations it is apparent that, in order to obtain a high yield of algae in locations where temperature varies in a wide range during the year, algal strains of different

[5] A strain of ordinary temperature-tolerance.

XUM

temperature-tolerance should be used according to the season. If, for example, in a climate such as that found in Tokyo, the strains given in Table III are used according to the season, we can expect an average yield of 12.4 gm./m.²/day throughout the year, or 18 metric tons of algae per acre per year. It may not be extravagant to infer that a culture plant located in a zone of favorable temperature with abundant sunshine would be able to produce an average of 20 gm. of algae per m.² per day, or 30 metric tons of algae per acre per year.

Mass Culturing of Nitrogen-Fixing Algae

It was pointed out by De (126) that in India and south-east Asia, where rice is grown without a frequent supply of fertilizer, the fertility of rice fields may be maintained, at least partly, by the activity of nitrogen-fixing blue-green algae. A far-reaching search for nitrogen-fixing algae was made by Watanabe (127 to 130) during the second World War. Out of more than 1000 algal strains collected from various regions of Asia, he found several strong nitrogen fixers, from which he selected *Tolypothrix tenuis* (from Borneo) as material for field experiments (131, 132). In laboratory experiments this blue-green alga grew (in the absence of a combined nitrogen source) at a rate of about 4 gm. (dry weight)/m.²/12 hr., fixing nitrogen at a rate of 0.24 gm./m.²/12 hr. This experiment was performed under rather unfavorable culture conditions, but it suggested that the alga can fix as much as 780 lb. of nitrogen per acre per year. Surprisingly higher values were recently found by Allen (49, 133) for *Anabaena cylindrica*, which was reported to grow, under favorable culture conditions, at a rate of 13 gm./m.²/12 hr., fixing in the meantime 0.9 gm. nitrogen/m.²/12 hr. This corresponds to a fixation of 2900 lb. of nitrogen per acre per year. For both *Tolypothrix* and *Anabaena*, the ability to support the growth of rice plants in the absence of combined nitrogen was demonstrated by pot-culture experiments (49, 129). Large-scale tests of the effect of *Tolypothrix* on the rice crop were started by Watanabe in 1951 at eleven experimental farms in various parts of Japan. The first problem he faced in this project was how to grow the alga in mass for distribution. It was found that *Tolypothrix*, unlike *Chlorella* and other common algae, requires aseptic conditions for its mass culture, serious contaminants being fast growing unicellular green algae and various other microorganisms which invade the culture even in the absence of combined nitrogen in the medium. The main reason for this seems to be that the alga excretes considerable amounts of nitrogenous and non-nitrogenous substances into the medium, a characteristic of many blue-green algae (14, 128, 134). In his procedure for aseptic mass culturing, Watanabe (131) used the following three techniques, of which the last mentioned deserves special attention for its originality and ingenuity.[6] (*a*) A stirring culture in a tank having

[6] In all these cultures Watanabe used a culture medium containing potassium nitrate as the nitrogen source. The presence of this salt increases the growth rate of *Tolypothrix* without decreasing its activity in nitrogen fixation.

glass windows through which the culture is illuminated from the outside (sterilized by heat). (*b*) The closed circulation culture [according to the principle of Arthur D. Little, Inc. (101, 102)] using a flat tube of vinyl sheeting (sterilized with hydrogen peroxide). (*c*) Culture on the moist surface of fine porous gravel[7] (2 to 4 mm. in diameter) soaked with nutrient medium (sterilized by heat). The inoculated moist gravel was kept in a tube of vinyl sheeting with illumination and aeration with CO_2-enriched air. After a certain period of culture, the gravel covered with algal cells was transferred to air-tight bags of vinyl sheeting and in this state either preserved or distributed to farms. When preserved in such a state, the algae were found to retain their growth activity almost unimpaired for as long as three years. The inoculation of paddy-field water was performed during the period from June to August[8] by seeding 130 to 650 lb. (fresh weight) of the "gravel culture" (containing 2 to 10 lb. dry weight of algae) per acre. Prior to seeding, the paddy water was sprinkled with lime powder which was effective in suppressing the growth of other algae and at the same time lowered the acidity of water to a favorable level (pH 7.5). One of the serious troubles encountered during the field experiments was the proliferation of daphnids which sometimes ate up the whole algal population within one or two weeks. This trouble could, however, be eliminated by sprinkling the field with an adequate quantity (0.05 p.p.m.) of a drug, "Folidol" (diethyl para-nitrophenol thiophosphate) (135). Despite these precautions, in some paddy fields (about three out of ten) *Tolypothrix* did not grow, the reason for the failure being obscure at present. However, in fields where the first seeding succeeded at all, seeding in subsequent years never failed, and although the seeding was made in the same quantity every year, there occurred a progressive increase of the crop year after year. The final steady-state increase in crop—both in N-poor and N-rich fields—is as much as 20 per cent, as may be seen from the figures given in Table IV. The effect of *Tolypothrix* in causing an increase of the rice crop was found to subsist in the field at least one year after the cessation of seeding. At any rate, these results make it evident that the practice of applying nitrogen-fixing algae to rice fields has great promise for the future. The performance of similar field tests using *A. cylindrica* is awaited with great interest.

Algal Culture in Sewage

It has been suggested by Caldwell (136) and Myers (137) that in sewage lagoons or oxidation ponds the stabilization of organic matter effected by aerobic bacteria is greatly aided by the simultaneous proliferation of algae. Organic matter is oxidized by bacteria using oxygen released by the photo-

[7] The gravel used is of special volcanic earth called "Kanuma-tsuchi" which is a weathered andesitic pumice containing volcanic glass, plagioclase, magnetite, hornblende, and hypersthene.

[8] This period extends from about a month before to about a month after the "bedding out" of young rice plants.

XUM

TABLE IV

PROGRESSIVE INCREASE OF RICE YIELD CAUSED BY *Tolypothrix tenuis* SEEDED ONCE A YEAR IN THE PADDY FIELDS*

	Percentage increase of crop compared with control					Yield of control culture per acre per year
	1st year	2nd year	3rd year	4th year	5th year	
N-poor fields†	4.6	11.2	14.6	19.6	22.9	1.2 metr. tons (42 bushels)
N-rich fields‡	1.1	8.3	20.3	20.2	—	2.1 metr. tons (73 bushels)

* From Watanabe (131, 132).

† Without addition of ordinary nitrogen fertilizer.

‡ 270 lb. of ammonium sulfate (containing 57 lb. of nitrogen) were supplied per acre per year.

synthesis of algae, which in turn, utilizing solar energy, synthesize organic matter from the carbon dioxide and ammonia produced by bacteria. The feasibility of such a symbiotic relationship has been extensively studied by Gotaas, Oswald and their coworkers (58 to 61, 96, 138 to 141) using oxidation ponds specially designed for the purpose. The ponds are equipped with automatic devices to control the rate of sewage inflow, liquid depth, and the rate of recirculation of effluent to seed the incoming sewage. The species of algae growing in the pond are *Chlorella*, *Scenedesmus*, *Chlamydomonas*, and *Euglena*, among which *Chlorella* is dominant in most cases [(58), see also Allen (142)].[9] The yield of algae as well as the degree of purification (or lowering of the BOD value)[10] of sewage water brought about by the algal-bacterial symbiosis depends on various factors such as (*a*) detention period of sewage in the pond, (*b*) depth of the liquid, (*c*) original BOD value of sewage (sewage strength), (*d*) bacterial and algal species, (*e*) amount of solar energy received, and (*f*) temperature. A rather simplified formulation relating these diverse factors has been proposed by Gotaas *et al.* (140, 141). According to their calculation, a suitable detention period is from one and one-half to four days

[9] According to Allen (142), who investigated the algal flora in sewage oxidation ponds now operating in several places in California, *Chlorella* (and, to a lesser extent, *Scenedesmus*) predominates at earlier stages of oxidation, but later, when oxidation is well advanced, it is succeeded by a mixed flora consisting of *Scenedesmus*, *Chlamydomonas*, *Euglena*, etc.

[10] BOD is the abbreviation of "biochemical oxygen demand" which is commonly used as a measure of the quantity of biologically oxidizable organic matter in liquid waste. It is expressed by the amount of oxygen (usually in mg. per l.) consumed by microorganisms during an initial 5-day period of incubation with the test liquid (at some constant temperature).

(depending mainly on the season and local climatological conditions), and a suitable pond depth is from 6 to 20 cm. (depending mainly on the sewage strength) (58). From their experimental results, Gotaas *et al.* inferred that a photosynthetic oxidation pond, when properly designed and operated, will bring about a BOD-removal of as much as 70 to 80 per cent, a light-energy utilization of as much as 5 to 8 per cent (139), and a production of algae in the amount of 30 to 35 tons/acre/year at Richmond, California, or 42 tons/acre/year at Phoenix, Arizona (140, 141). One technical problem, which seems important in determining the economic feasibility of this project, is the method of harvesting algal cells from the sewage. Whereas in the mass culture of green algae discussed in earlier chapters the density of algae in the culture may be raised to a level as high as several grams per liter, there is a rather sharp upper limit of algal density attainable in the culture in sewage. This limit, which is determined principally by the carbon and nitrogen contents of sewage, is only about 0.3 gm. per l. in moderately strong domestic sewage (140). Present-day centrifuge equipment does not appear to be satisfactory from an economic standpoint for separating the algae from a liquid of such low density. To circumvent this difficulty, Gotaas *et al.* (58) proposed a method of precipitating algal cells with alum as a coagulant. The feasibility of separating and recovering aluminum floc on a large scale is a problem which must await further experimentation.

The use of sewage water as a medium for growing *S. obliquus* was studied by Meffert (111) using the open bubbling culture method. The culture conditions differed from those of Gotaas *et al.* in that carbon dioxide was supplied by introducing methane gas, containing 30 per cent CO_2, which was produced also by the treatment of sewage. The multiplication of algal cells occurred actively in the first few days (yield, 4.4 to 11.6 gm./m.^{2}/day; efficiency of energy-conversion, 1.3 to 2.9 per cent), but then decreased for lack of nitrogen, leading to a decrease of protein content in algal cells. As a result of algal-bacterial proliferation both the organic matter (as measured with potassium permanganate) and ammonia-N in the medium decreased considerably.

Utilization of Algal Cells and Economic Appraisal

The potential use of mass-cultured green algae as sources of protein, fat and vitamins, or as starting materials for organic syntheses, has been discussed from various angles in the Carnegie Monograph (8). The animals for which *Chlorella* or *Scenedesmus* have been shown to have good nutritive value are the rat (44, 143), the chick (140, 144, 145, 146), the larvae of some mollusks (117, 147 to 151), and daphnids (152). Worthy of special attention is the recent work of Fink (143) who tested the nutritive value of the protein of *S. obliquus* using albino rats as test animals. The digestibility of the protein of dried algal cells was found to be 60 per cent, as compared with 80 per cent for milk protein. The nutritive value of algal protein was, however, surprisingly high, being comparable to, or even superior to that of dried skim

XUM

milk or egg white (see also 153, 154). Fink noticed that in his many years' study of feeding rats with microbial proteins (yeasts, molds, mushrooms, etc.), none was so high in nutritive value and none was eaten with such pleasure by animals as algal protein. Moreover, it was remarked that during the 120 days' feeding experiments not one animal perished when fed the algal diet, while with the control diets (skim milk) a high percentage of animals died, the cause being in most cases the so-called dietetic liver necrosis. This result indicates that some substance which is effective in preventing or healing dietetic liver necrosis is contained in algal cells. The nutritive value of *Chlorella* or *Scenedesmus* for humans has not yet been tested, except, in a rather crude way, by Jorgensen *et al.* (155) who obtained a favorable result with "plankton soup"—consisting largely of green algae including *Chlorella* and *Scenedesmus*—fed to human patients in a Venezuelan leper colony. Tests of the palatability of *Chlorella* were performed by Morimura *et al.* (104, 156) by adding dried algal powder in varying concentrations to various foods such as bread, noodles, soups, ice cream, and "miso" or fermented soybeans. Comparative tasting by a number of American and Japanese people showed that, by and large, the *Chlorella*-containing foods appealed more to Japanese than to American people, which may be attributed to the fact that the former are accustomed to foods including sea-weeds and other vegetables which are similar to *Chlorella* not only in appearance, but also in odor and taste.

For the production of algae at a reasonable price a culture plant of a fairly large scale must be envisaged. Assuming a plant covering 100 acres of land, estimates of the production cost of *Chlorella* have been made by Fisher (102, 103) and by Tamiya (104). Supposing that a yield of 32 tons/acre/year could be obtained by the closed circulation system, Fisher estimated the cost of dry *Chlorella* to be 17 to 25 cents per lb., while Tamiya, assuming a yield of 18 tons/acre/year from an open circulation system, obtained the figure of 26 cents per lb. The price of algae grown in sewage oxidation ponds has not yet been estimated; obviously it must be considerably lower than those given above. There are no data available as yet for estimating the cost of nitrogen-fixing blue-green algae. The special consideration in this case is that the algae grown in the plant will be used only as seeding material, the substantial increase of algae occurring later in the paddy field. Supposing that the algae initially seeded in the amount of 2 lb. (dry weight) per acre will increase 200-fold during the year, fixing 24 lb. of atmospheric nitrogen, 1 lb. (dry weight) of seeding algae will be equivalent to 57 lb. of ammonium sulfate. Since this amount of ammonium sulfate costs $1.47 (U.S.) (the wholesale price in Japan), these algae may be produced at a considerably higher cost than is estimated above for *Chlorella*.

General Comment

As we have seen, the feasibility of algal mass culture has been proven by many experiments performed during the past several years. In view of the

progress made both in theory and practice during this period, it seems justified to say that the time will not be far distant when the industrialization of algal mass culture will be realized. The significance of the effort to attain this aim is indubitable when one considers the ever-increasing pressure of population and the existence of untold millions of undernourished people in the world. Equally evident is the significance of basic studies underlying the project of algal mass culture as an effective avenue of approach to the understanding of the growth process of green plants in general. Recently some criticism has been raised against the enthusiasm of investigators of algal mass culture, pointing out that they are over-sanguine about the efficiency of photosynthetic activity of algae (cf. 157, 158). It is true that in the past there were some articles which foretold far-fetched visions about the "marvels" of *Chlorella*. "Vision is necessary, but the vision must be a disciplined and practicable one" [Spoehr (2)], and it is the hope of the present writer that this review may show that the study of algal mass culture is now on a sound and right track, and that the arguments of its opponents are unfounded.

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XUM

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PERMEABILITY OF PLANT CELLS[1]

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Permeability and Active Transport

This review will deal solely with permeability in the restricted sense of the word. The phenomena of nonosmotic, active, or metabolic transport, thus, fall outside its scope. Nevertheless, it may not be out of place to consider, by way of introduction, to what extent the flow of different substances through plant protoplasts is caused by metabolic factors and to what extent it is purely osmotic in nature.

Regarding inorganic ions, it is now universally realized that their uptake is governed mostly by metabolic forces. The same seems true for the absorption of such strongly ionized organic compounds as, for instance, the sulfonic acid dyes [Drawert & Endlich (21)] and streptomycin [Pramer (54)]. A nonosmotic uptake of sugars and amino acids is also, at least in some cases, fairly convincingly established [cf., for instance, Rothstein (59) and the papers contained in volume VIII of the *Symposia of the Society for Experimental Biology* (66)]. There are, however, other substances whose uptake—whether by simple permeation or active transport—is still an open question. One of the most vigorously discussed cases is that of water. Here, however, it is not necessary to enter into the arguments for and against the active transport of water, since Kramer (45, 46) recently published two excellent surveys of this question. The present reviewer agrees entirely with Kramer's view that, although it has been clearly demonstrated that water uptake is in many cases reduced or even prevented by oxygen deficiency and respiratory inhibitors, and increased by auxin, this is not conclusive proof that respiratory energy is being used directly to bring about the intake of water. A considerable proportion of the supposed cases of nonosmotic water transport may in reality be due to indirect effects. For instance, it is clear that all factors which affect growth must at the same time affect water uptake since it constitutes an essential part of the growth process. Kramer does not believe that nonosmotic absorption of water is an important factor in plant cell water relations, but considers it may possibly occur to a limited extent. The papers of Bogen (13), Pohl (53), Prell (55), and of Lott & Rosene (48), which have appeared since Kramer wrote his surveys, have scarcely invalidated his views, while those of Levitt (47), Ordin & Bonner (51) Ordin *et al.* (50), Bonner *et al.* (15), and Thimann & Samuel (67) tend to lend even more support to his

[1] This subject was last reviewed in this series by Virgin in 1953. Certain aspects of it were treated also by Kramer (45) and by Epstein (22). Volume II of Ruhland's monumental *Encyclopedia of Plant Physiology* (61) which will, no doubt, include a thorough treatment of the permeability of plant cells, had not yet appeared when the present review was concluded in August, 1956.

XUM

critical attitude towards the alleged general occurrence of active water transport.

At present, the majority of investigators seem to be of the opinion that the substances capable of being actively transported all fall within a fairly limited number of chemical categories: salts, sugars, amino acids, etc. On the other hand, Bogen and his coworkers maintain the view that metabolic forces materially affect the flux of a very great variety of quite dissimilar substances through plant protoplasts. Some of their most striking results were achieved in experiments with diatoms. For a fairly long time it has been known that if diatoms are plasmolyzed in solutions of sugars, polyhydric alcohols, amides, or other substances generally acknowledged as slow permeants, recovery from plasmolysis will occur with a rapidity which is several times greater than that encountered in other cells under similar conditions. So far, this has been interpreted as indicating an unusually great permeability of diatoms to the solutes tested. Bogen & Follmann (14) found, however, that metabolic inhibitors such as azide or dinitrophenol greatly depress the deplasmolysis rate of the diatoms. They, therefore, assume that the recovery from plasmolysis is brought about by four simultaneous processes, viz., (*a*) simple permeation of the solute, (*b*) active uptake of the solute, (*c*) osmotic uptake of water, and (*d*) nonosmotic water uptake. The active uptake of the solutes tested is calculated at about 30 to 80 per cent of their total flux. The generally presumed unusually great permeability of the diatoms is thus, according to Bogen & Follmann, nonexistent [cf. also Follmann (24)]. Moreover, although active transport seems especially striking in the case of the diatoms, Bogen and his associates have tried to show that other plant protoplasts behave in an essentially similar way [Bogen (11, 12, 13); Prell (55)]. Many of their results are, in fact, very peculiar and present a marked contrast to the experiments of, for instance, Bärlund and Collander. A critical re-examination of the whole subject seems, therefore, unavoidable. An investigation of Johnson & Bonner (37) shows how even one and the same substance (2,4-phenoxyacetic acid) may be taken up, or bound, in several different ways by a single kind of tissue (the *Avena* coleoptile parenchyma).

According to Danielli (20) there is, besides osmotic and nonosmotic uptake, yet a third type of absorption which he calls facilitated diffusion. It resembles simple permeation in so far as both occur under the driving force of thermal agitation and both reach the same equilibrium. On the other hand, facilitated diffusion is restricted both by structural and steric factors and is thus pronouncedly specific in nature. So far, however, the occurrence of facilitated diffusion in plant cells does not seem to have been definitely established.

Sites of Resistance to Penetration

Evidently in its passage from the medium to the cell sap, every permeating substance has to overcome four successive diffusion resistances, namely those of (*a*) the cell wall, (*b*) the outer plasma membrane or plasmalemma,

(*c*) the bulk protoplasm or mesoplasm, and (*d*) the inner plasma membrane or tonoplast.

Of these four resistances the first has received comparatively little attention. In most cases it may well be relatively insignificant. The experiments of Horié (34) show, however, that even the cell walls of a submerged green alga, *Hydrodictyon*, have a rather restricted permeability, while Biebl (10) gives impressive examples of the great permeability differences between the cell walls of closely related moss species. Besides, several water-soluble polysaccharides and other large-molecular substances are able, when introduced into the transpiration stream, to block the intermicellar spaces in the cell walls and thus cause wilting of the plant. Some wilt toxins produced by pathogenic fungi owe their harmful effect to this fact [Kern (39)].

The resistances (*b*) to (*d*) are, physiologically, of greater interest, but in spite of this no exact data concerning either their absolute or their relative magnitudes are so far available. The classical view represented by Pfeffer and Overton, among others, was that the diffusion resistance of the mesoplasm is always small while both the outer and the inner plasma membranes are more or less "semipermeable" and thus offer a very great resistance to the diffusion of numerous solutes. In recent years this view has often been criticized. In particular, it is now assumed in many quarters that the plasmalemma is much more permeable, at least to ions, than the tonoplast [Arisz (2)]. Ideas of this kind are reflected in recent views concerning the nature of the "apparent free space" (A.F.S.) or "outer region," i.e., that portion of a tissue to which solutes apparently move by free diffusion [cf. Epstein (22)]. It is now often assumed that the A.F.S. consists not only of cell walls and intercellular spaces but that it includes at least parts of the protoplasm also. If this assumption is correct, then it would, of course, follow that the plasmalemma must be readily permeable to numerous solutes which only very slowly penetrate the tonoplast. As can be seen from the report of Epstein, there are, in fact, several observations which seem to sustain such views. On the other hand, it should not be forgotten that the test objects on which these studies have been carried out—mostly roots of higher plants —are very complicated structures consisting of epidermal, cortical, and endodermal cells, vessels, etc. Moreover, the protoplasm constitutes only a rather small fraction of the total tissue volume. Thus, it is doubtful whether these results, although suggestive, are really accurate enough to counterbalance the numerous facts and evidence in favor of the classical concept of a very restricted permeability of the outer plasma membrane.

Bennett & Rideal (9) make the plausible suggestion that, in *Nitella* cells, the protoplasm/external solution interface lies within the interstices of the cell wall, and that its area is, therefore, only a fraction of the total cell-surface area. "It is not known how far the protoplasm penetrates into the interstices of the cell wall, but since acid fuchsin (a water-soluble dye which does not stain living protoplasm) does not pass into the wall when placed in the external solution, it seems most likely that the protoplasm comes near,

XUM

but not actually up to, the outer surface of the cell wall." Upon plasmolysis, fine strands of protoplasm are left clinging to points on the inside of the wall, with a resultant great increase in the area of the interface between the protoplasm and the external solution. The tonoplast, on the other hand, has probably not changed to anything like the same extent. Experimentally it was found that the electrical resistance of the system falls and the capacity rises sharply when plasmolysis sets in. These observations, too, suggest that the resistance of the outer plasma membrane is by no means negligible as compared with the resistance of the inner one.

Hofmeister (33) found the vegetative cell of the pollen grain devoid of a plasmalemma. Its protoplasm is therefore miscible with water. The generative cell, on the other hand, is endowed with a normal plasmalemma.

Permeability to Nonelectrolytes

In order to clear up the still somewhat controversial problem of the stucture of the plasma membranes and the permeation mechanism, investigations are desirable in which the permeability of some selected types of cells towards as great a multitude of substances as possible is studied. It is obvious that the more varied the combinations of physical and chemical properties of the substances tested, the better founded will be the conclusions. An investigation with such an object in mind has been carried out with *Nitella* cells [Collander (16)]. In its general outline it resembles an earlier investigation of Collander & Bärlund on the cells of *Chara ceratophylla*. This time, however, the number of substances tested was somewhat greater. The new investigation also covers a somewhat greater range of permeation power, in that the most rapidly permeating substance (heavy water) had a permeation power roughly 10,000,000 times greater than the slowest one (pentaerythritol). In the great majority of cases, the substance to be tested was first permitted to saturate the cell, whereupon its efflux rate was determined. Any considerable intervention of metabolic forces in these experiments seems improbable. The permeation powers of the substances tested were found to be roughly proportional to $k^{1.3}$, where k denotes the partition coefficient ether/water or, still better, olive oil/water. At the same time they are, to a first approximation, inversely proportional to about the 1.5th power of the molecular weight. The permeation power of the very smallest molecules is greater, however, than these statements imply. It should be noted that the negative correlation found between molecular weight and permeation power does not prove that the molecular size as such is a factor directly concerned with the permeation process. Similarly, the empirically found correlation between permeation power and lipoid solubility does not constitute conclusive proof of the view that the permeants enter or leave the protoplasts by truly dissolving in the plasma membrane lipoids. It does indicate, however, that the permeation process consists of events which are at least very closely connected with solubility. In fact, the penetration of molecules through the plasma membrane is probably primarily controlled by in-

termolecular forces identical with those which control the partition of a solute between two mutually immiscible solvents.

A remarkable result of the comparative study of the permeability properties of different plant cells is the observation that while, on the whole, the permeability of different cells is in most respects fairly uniform, the ratio $P_{urea}/P_{methyl\ urea}$ is subject to strikingly great variations [cf. Virgin (73)]. Wartiovaara & Tikkanen (76) have tried to gain a deeper insight into the causes underlying these variations by determining the temperature dependence of the permeation of urea and some of its derivatives. The temperature coefficient (Q_{10}) of the permeation determined for five very different types of plant cells was found to vary between 1.9 and 2.4 for urea and thiourea and between 3.2 and 3.8 for methyl and dimethylurea. The magnitude of the Q_{10} seems thus remarkably independent of the cell type, and also of the permeation power, but closely dependent on some other properties of the permeating molecules. An exhaustive explanation of these findings is scarcely possible at present, but the authors suggest that the steric properties of the permeating molecules may be the deciding factor. This view is supported by Url (69), who points out that acetamide has about the same molecular weight as urea (66 versus 60, respectively) and propionamide about the same as methyl urea (79 versus 74). A priori, it would therefore seem plausible to assume that just as the ratio $P_{urea}/P_{methyl\ urea}$ exhibits great variations, so the same would also be the case regarding the ratio $P_{acetamide}/P_{propionamide}$. In reality, however, this is not so: Url found, in conformity with earlier results, that propionamide always permeates more rapidly than does acetamide.

Finally, some new results on the permeability to water may be mentioned. Owing to technical difficulties there are still only a few reliable measurements of its magnitude. A promising technique used with single *Nitella* cells has been developed by Kamiya & Tazawa (38). The two ends of the cell are brought in contact with external solutions having different osmotic pressures, e.g., water at one end and a sucrose solution at the other. Under these circumstances a flow of water through the cell will be induced and the rate of this flux can be very exactly measured by means of the double-chamber volumeter previously developed by Kamiya. In calculating the permeability of water, allowance must be made for the polarization brought about by the sap inside the cell flow which counteracts the osmotic gradient in the external medium. A remarkable result attained with this method is that the permeability to water was found to be distinctly greater in the inward direction than in the reverse one. Besides, the water permeability, expressed in μ/min./atm., was found greater than in any previous osmotic experiments.

The technique of Kamiya & Tazawa has the special merit of not altering the physiological state of the cells examined in that all drastic operations, such as plasmolysis or deplasmolysis, are avoided. The same is true of a new method developed by Virgin (74) with which even small changes in the turgor of plant tissues, or of single giant cells, can be detected. His method

XUM

is based upon the fact that a solid body, which is caused to vibrate, will oscillate with a certain frequency which will change when the rigidity or mass of the body is changed. Measurements of water uptake and water loss can thus be advantageously made with this method.

Prescott (56) found activated salmon eggs "completely impermeable" to water. Although referring to a zoological object, this result is so exciting that it seems justifiable to mention it in this review otherwise devoted to plant cells alone.

Permeability to Salts and Ions

The permeability of plant cells to salts and ions, as well as the active transport of ions by them, has been excellently reviewed by Epstein in the last volume of this series (22). A few additional remarks on this subject will therefore suffice here.

According to Roberts *et al.* (58) the cells of *Escherichia coli* are so permeable to all noncolloidal substances (e.g., sodium, potassium, manganese, sulfate, phosphate ions, and several amino acids) that equality of concentration with the ambient solution is in all cases reached within 5 min. (With the techniques used, shorter time intervals could not be tested.) In emphasizing the extreme permeability of these cells, the authors even resort to such a drastic metaphor as this: "The protoplasm (of *E. coli*) may be likened to a sponge, the cell membrane to a surrounding hair net." Similar, although less detailed, statements are also given for a yeast, *Torulopsis utilis*. The first impression is that the permeability properties of the two organisms in question differ quite radically from those of ordinary plant cells. In evaluating these findings it must not be forgotten, however, that the surface/volume ratio in such minute cells, is, of course, much greater than in "normal" plant cells. Thus, assuming that the permeation constants are the same for ordinary plant cells as for *E. coli*, the same amount of exchange would demand, roughly, as many hours with ordinary cells as minutes with the bacteria. But even taking this into account, the permeability of the *Escherichia* and *Torulopsis* cells, as described by Roberts *et al.*, seems somewhat unexpectedly great. At least it deviates decidedly from the results obtained by numerous investigators [Äyräpää (4), Conway & Downey (18), Conway & Kernan (19), Rothstein & Hayes (60)] with baker's yeast. Moreover, Collander (17) found the permeability of an unidentified luminous bacterium great but not excessively so. The salt permeability of this bacterium seemed to be of the same order of magnitude as its permeability to such relatively slowly permeating nonelectrolytes as, say, sucrose or raffinose. Just as with ordinary plant cells, ions were found to permeate much more slowly than even large molecules of lipoid-soluble substances, such as alkaloids. Besides, Fricke *et al.* (27) found that *E. coli* possesses a low-conductance surface membrane of approximately the same thickness and dielectric constant as is found in other cell species. Thus it seems at any rate obvious that the "hair net concept" should not too quickly be accepted as valid for bacteria and yeasts in general.

Arisz (3) has, on the basis of the symplasm theory, given an interesting picture of the transport of salts and other substances in multicellular plants.

Permeability to Weak Acids and Bases

It has long been known that the physiological activity of weak acids and bases is greatly influenced by the pH of the medium in which they are applied to the cell. In general, the activity of weak acids is highest in acid media and that of weak bases in alkaline ones. That this must be so is easily understood if we assume (*a*) that the site of the physiological effects studied is in the interior of the cytoplasm, not on its outer surface, and (*b*) that the outer plasma membrane is permeable to the undissociated acid and base molecules but more or less impermeable to the corresponding ions. Simon & Beevers (63) have subjected this question to an extensive re-examination. Their results, on the whole, corroborate earlier concepts, with one remarkable exception, however: the physiological activity of weak acids is consistently somewhat greater than would be expected at very high pH values, and so also is that of weak bases at very low pH values. Whether this implies that the ions, too, have an appreciable permeation power is, however, very doubtful.

The view that the permeation power is, primarily at least, related to the undissociated acid and base molecules is supported by the recent investigations of Bennett (8) on the toxicity of growth substances to *Nitella*, of Foulkes (25) on the uptake of pyruvic acid by yeast, of Erickson *et al.* (23) on the influence of acetic acid and dichlorophenoxyacetic acid on *Chlorella*, of Slonimski (64) on the entrance of benzimidazole into yeast cells, of Aldous & Rozee (1) on the toxicity of fluoroacetic acid to yeast, and of Macmillan (49) on the entry of ammonia into fungal cells. Bennett remarks, however, that his results are understandable only if we assume that the pH at the surface of the plasma membrane is about 1.1 units lower than the pH value in the bulk solution. This observation is consonant with Danielli's statement that the differences between bulk and surface concentration of hydrogen ions may be of the order of several pH units, and hence might be of great importance in biological processes.

Kral (44), using a technique originally devised by Jacobs, measured plasmometrically the permeability of *Tradescantia* cells to acetic acid, ammonia, and several alkyl amines. If the permeation constant of ammonia is arbitrarily put equal to unity, those of the other bases tested acquire the following mean values (line I below):

	Ammonia	Methylamine	Ethylamine	Dimethylamine	Trimethylamine	Diethylamine	Triethylamine
I	1.0	2.0	1.1	10.6	3.5	3.3	2.7
II	1.0	0.3	0.1	0.2	0.1	0.6	0.2

Line II of the above compilation contains the relative permeation constant values found by Äyräpää (4) for yeast cells. It is seen that while Äyräpää, in accordance with the earlier less accurate findings of Poijärvi on cells of

XUM

higher plants, found the permeation power of ammonia to be greater than that of the amines mentioned, Kral found an inverse relation. The causes of this discrepancy are not discussed by the author.

Suomalainen & Oura (65) stress that it is impossible to control the intracellular pH of yeast cells by the conventional phosphate, citrate, or succinate buffers, while acetate and ammonia buffers, for instance, are well suited for such a purpose. To a cell physiologist, acquainted with the permeability properties of normal plant cells, this comes as no surprise, but to many a biochemist this paper may serve as a wholesome warning. Other applications to biochemical problems of our knowledge concerning the permeability of cells to acids and bases are given in the papers of Beevers *et al.* (7) and of Kinzel & Url (43). The former point out that where cells are found impermeable to acids or bases to be introduced into living cells, some corresponding esters may be profitably used for this purpose, while the latter stress that in catalase determinations it is not always enough to disintegrate the tissue to be studied in a conventional buffer solution: a readily penetrating base such as ammonia must often be used in order to prevent destruction of the highly susceptible enzyme by the acids of the cell sap.

Waris (75) tested some 40 weak acids with regard to their physiological and toxic effects on *Micrasterias* cells. Variation of the permeation power was, however, only a minor factor in this study.

Permeation of Dyes

In spite of the frequent use of dyestuffs for permeability studies, our present knowledge concerning the permeation power of dyes is still of a highly nonquantitative, and thus, unsatisfactory character. In a very few cases only have the amounts of dyes taken up in a given time by cells of known dimensions been quantitatively determined, and extremely seldom has the magnitude of the concentration gradient causing the dye uptake been known at the same time. Thus, only in quite exceptional cases has it been possible to evaluate even the order of magnitude of the permeation constant of a dye. An investigation of Bartels (5) constitutes an important step forward. His careful microspectroscopic measurements of the amounts of a basic dye, acridine orange, taken up by the epidermal cells of *Allium*, surpass all earlier measurements of this kind. The permeability constant found by him, about 1×10^{-4} cm./sec., is, therefore, probably the most accurate permeation constant hitherto determined for a basic dye. This statement holds true whether or not all of his theoretical interpretations are quite to the point [cf. Kinzel (41)]. Another similar investigation is that of Bartels & Schwantes (6) on the uptake of thionine by the same cells. A fairly simple microcolorimeter for the determination of the amounts of dye taken up by cells of microscopic dimensions has been devized by Kinzel (42).

Höfler, in collaboration with several other members of the Vienna plant

physiology school, has published a long series of papers on vital staining with basic dyes. A prominent feature of these studies is their comparative trend; they are based on experiments with a great multitude of different plant cells including cells of numerous lower plants, especially the algae. The purpose of these studies is not only the elucidation of the permeability of these cells towards dyes but also, and perhaps even more an analysis of the various mechanisms by which the dyes may be accumulated in the cells. Most of their essential results have been summarized in two recent publications by Höfler (30) and by Höfler & Schindler (31).

The investigations of the Vienna school have corroborated the view that the plant protoplasts, while readily permeable to most kinds of dye base molecules, are at the same time impermeable to the dye cations. Nevertheless, Höfler (29) found that the tonoplasts of several plant cells are permeable to toluidine blue cations. This observation is of great interest, especially in view of the great gaps in our present knowledge concerning the permeability properties of the tonoplast. The only possible objection to be raised against the general bearing of this discovery is that the normal state of the tonoplasts studied was not definitely established.

As much as 10 years ago, Höfler distinguished between two main types of accumulation of basic dyes in the sap of plant cells. In one the accumulation is simply due to the acidity of the cell sap: dye base molecules which have entered the sap are here converted into nonpenetrating dye cations, which are thus trapped in the sap, while more dye base continues to enter the sap as long as the concentration of the undissociated dye base molecules is lower in the sap than in the surrounding solution. The second type, on the other hand, is characterized by the occurrence of some other substances in the sap capable of binding the dye. Cell saps of the first-named type are called by Höfler "empty" (German: leer); those of the second type "full" (German: voll). Saps giving a precipitate with entering dyes constitute a third type. As shown by Höfler and his co-workers, there exist a large number of microscopically demonstrable differences between the empty saps, on the one hand, and the full ones, on the other. (*a*) Many dyes exhibit different colors according to the quality of the sap. Thus neutral red appears about brick-red in empty, but more pink-red in full saps, while acridine orange has a green fluorescence in empty, but a red one in full saps. (*b*) When treated with dilute ammonia solutions, cells rapidly lose the dye accumulated by them if the sap is of the empty type, while saps of the other type remain unaltered or nearly so. (*c*) Extremely weak dye bases, such as rhodamine B, are unable to form salts even in the most acid cell saps. Such dyes are therefore accumulated by full saps only. (*d*) Cells of the full sap type are able to accumulate basic dyes even from solutions of comparatively low pH, while cells of the other type demand higher pH values. (*e*) Vacuolar contraction is very often caused by basic dyes if the sap is empty, but seldom if it is full. The fairly simple trap meachanism causing accumulation of basic dyes in

XUM

cells with empty sap has been thoroughly elucidated in a quantitative respect by Kinzel (40).

Structure of the Plasma Membranes and the Theory of Their Permeability

A notable, and very gratifying, trend in the present-day discussion on the theory of cell permeability is that the harsh antagonism between different schools, so characteristic of discussions on this subject some 20 to 30 years ago, has been largely overcome. It is now almost universally recognized that lipoid-solubility (or some quality closely correlated with it) is a factor of prime importance in determining the permeation power, but that the permeation is also in some way influenced by the molecular size of the permeants (or by some quality correlated with it). The problem of today seems thus mainly to be to find out just to what extent, and in what manner, these two factors govern the permeation process.

A question vigorously discussed in this connection is whether the plasma membranes contain "pores" or not. Like many other puzzling question, this, too, seems to be largely a question of definition. In what case shall we then designate a plasmatic membrane as endowed with pores? Ussing (70, 71, 72) gives a practical answer to this question: if the water permeability of a cell membrane as measured by the rate of osmosis proves higher than its water permeability as measured by the rate of diffusion of isotopic water, then it seems appropriate to speak of a bulk flow of water through pores in the membrane. Such a result indicates, according to him, that the Poiseuille flow through pores dominates over the diffusion of water [cf. Pappenheimer (52)]. Ussing points out, however, that the "pores" revealed in this way "need not be permanent structures, but may form and close continuously." Moreover, it is doubtful whether it is possible with living protoplasts to compare "osmotic" and "isotopic" water permeabilities exactly enough to make judgements concerning the occurrence or absence of a Poiseuille flow across them [cf. Jacobs (36, p. 156)]. Whether plant cell membranes are equipped with pores in the Ussing sense does not yet seem to have been investigated, but for animal cells some pertinent data have been published by Prescott & Zeuthen (57).

A stimulating contribution to the discussion concerning the structure of the plasma membranes is given by Frey-Wyssling (26) in his latest book on the submicroscopic structure of the cytoplasm. Referring to some electron microscope pictures by Houwink (35) and Salton & Williams (62), he assumes that the plasma membrane is composed of regularly arranged globular macromolecules, mucoproteidic or rather lipoproteidic in nature. He further suggests that in the plasma membrane the lipoid chains fill up most of the interstices left between the globular macromolecules. Frey-Wyssling points out that his views resemble those underlying the old mosaic theory, except that protein and lipoid areas are now considered to be of molecular dimen-

XUM

sions only. A consequence of this is, he says, that stoichiometric relations between these components are possible, so that definite lipoproteids may occur in the membrane. He stresses that in his view the lipoids do not constitute a particular layer of their own, but occur only as intermolecular or intramolecular components between the globular protein molecules, at the same time both stabilizing the membrane and limiting its permeability. (Incidentally, the hypothesis of Frey-Wyssling seems to be a very accurate equivalent to Overton's more sweeping formulation of "a membrane impregnated with lipoids," translated into modern submicroscopic terminology.)

The present reviewer is unable to say just how probable, from a physicochemical point of view, a structure like that suggested by Frey-Wyssling would be, but it seems to him obvious that a membrane of such a structure, if really existing, would, indeed, have permeability properties very similar to those characterizing living protoplasts. Regrettably, however, it seems that the empirical basis of this hypothesis is somewhat weak in so far as Houwink and Salton & Williams, both designated, probably correctly, the structures photographed by them, not as plasma membranes, but as cell walls.

Umrath (68) maintains the view that in plasmolyzed or otherwise injured cells a new plasmalemma is produced by a precipitation process in which calcium ions play an important role. There are, however, according to him, two alternative paths given for the process. If (*a*) the external solution is relatively rich in calcium ions the precipitate will be formed from external calcium ions plus anions coming from the protoplasm. If, however, (*b*) the external medium contains no calcium ions but instead some calcium precipitating anions, the precipitate owes its origin to the combination of protoplasmic calcium ions with external anions. This concept is based, among other things, on the observation that certain plant cells will withstand plasmolysis in pure potassium oxalate solutions while, strangely enough, plasmolysis in a potassium chloride solution to which a trace of potassium oxalate has been added proves more injurious. According to Umrath the plasmalemma is, in the pure oxalate solution, produced according to alternative (*b*), while in a solution containing only a very low oxalate concentration neither reaction (*a*) nor (*b*) will occur.

Hofmeister (32) showed experimentally that under most conditions it is very difficult to make isolated plant protoplasts fuse with each other. Only after treatment with certain reagents, e.g., detergents, will such a fusion occur more readily. The results suggest that polypeptide chains occurring in the plasmalemma normally prevent the fusion.

An exact knowledge of the magnitude of the tension at the protoplasmic surface is of considerable importance as a basis for evaluating the plausibility of the different hypotheses concerning the plasma membrane structure proposed by different authors. The brilliant survey of this question recently given by Harvey (28) will therefore prove of great value in this respect.

XUM

Permeability Changes

For about half a century there has been a continuous flood of statements concerning permeability changes brought about by a great multitude of factors and supposed to serve many different purposes in the life of the plant. Unfortunately, most of these statements are rather inadequately supported by experimental facts. But perhaps it is not too optimistic to say that in the last few years the first signs of an abatement of this flood are recognizable. At any rate, such a development would now seem only logical, since it has been generally realized that active transport, rather than permeability proper, is, in most cases, a factor not only dependent on, but also itself influencing, the rate of metabolic processes.

The space available here does not permit an analysis of individual papers concerning permeability changes. Besides, Kramer (45) has recently published an account of some work in this field.

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PHYSIOLOGY OF PHLOEM[1]

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The present article is concerned mainly with the newest literature on phloem physiology, especially with those contributions that either were not reviewed or were incompletely considered in the most recent compilations on this subject [Arisz (2); Crafts (24)]. The article emphasizes the relation between structure and function of the tissue elements and omits many of the physiological studies on translocation that seem to contribute no new information regarding this relation. The movement of growth-regulating substances is not considered at all because this topic was just summarized in this publication [van Overbeek (98)].

Before one considers the latest studies on structure and function of the phloem, the main hypotheses of translocation should be recalled. As is well known, translocation of organic substances is regarded as the principal function of the phloem tissue, and the enucleate sieve element is generally assumed to be the main conduit of the translocated materials. The nucleate parenchymatous elements of the phloem, or at least some of them, are thought to be concerned with translocation in a still undetermined manner. Parenchyma cells undoubtedly are involved also in various other activities characteristic of living cells, notably the alternate storing and mobilizing of food materials.

Most of the studies on phloem physiology deal with translocation. Workers agree that movement of materials in the phloem occurs at rates greater than those observed in ordinary parenchyma cells, but the mechanism of this movement is a perennial subject of controversy. The main disagreement in the interpretation of this mechanism is intimately related to the diverging concepts of the nature of the sieve-element protoplast and its role in translocation. The various diffusion hypotheses of translocation, including the hypothesis involving cytoplasmic streaming, assume that the cytoplasm of the sieve element is actively concerned with the movement of materials, or that, at least, the solutes are transported in the cytoplasm or along its surfaces independently of the water and independently of each other [e.g., Schumacher (87)]. The pressure-flow hypothesis, on the other hand, visualizes a mass movement of assimilates and water through the lumina of sieve elements and across the walls between elements [e.g., Crafts (19, 20, 23)].

Thus the cytophysiology of the sieve elements is of the most immediate interest with regard to translocation. In this connection, the structure of the sieve areas—characteristic of the sieve elements—needs special atten-

[1] The survey of the literature pertaining to this review was concluded in June, 1956.

XUM

tion. Finally, the role of the various parenchymatous cells of the phloem must be revealed before the function of the phloem is properly understood.

Sieve-Element Protoplast

The peculiar characteristics of the sieve-element protoplast are undoubtedly related to the physiologic specialization of these elements. A comparative study of food-conducting cells in different groups of plants suggests that sieve elements evolved from parenchyma cells and that one of the earliest obvious changes must have been related to the enzymatic components. This change is indicated by the consistent absence of ordinary starch—the so-called sieve-tube starch stains red with iodine [Esau (32)]—in sieve elements of vascular plants. Such absence has been noted even in the leptom cells of the moss *Polytrichum* [Esau *et al.* (34)] that are interpreted as food-conducting cells in this plant. In the mature sieve elements the scarcity of stainable material and the absence of nuclei are the other two well-known visible expressions of specialization of the protoplasts of these elements that set them apart from parenchyma cells.

The relation of these unique cytologic features to the activities within the sieve elements is not understood. The proponents of the diffusion hypotheses of translocation tend to disregard the peculiarities of the sieve-element protoplast since, as mentioned, they visualize the cytoplasm as actively participating in the movement of materials. The mass-flow hypothesis —in some of its versions at least—includes the concept that the sieve-element protoplast is denatured in the sense of having lost its property of differential permeability [cf. Crafts (20, 21, 23)].

An accepted test for differential permeability is the response to plasmolytic agents. In this regard two diverging sets of data were originally presented in the literature; Crafts (20, 21) reported inability to plasmolyze mature sieve elements, but Schumacher (85) described successful plasmolysis and deplasmolysis in sieve elements. Subsequent studies by Rouschal (78) and by Currier *et al.* (25) showed that repeated plasmolysis and deplasmolysis can, indeed, be induced in mature enucleate elements. These workers, however, emphasized that the sieve elements are sensitive to injury and, if not carefully handled, cease to be plasmolyzable. Currier *et al.* (25) found that measures designed to reduce sudden changes in environment increased the number of plasmolyzable elements in sections.

Currier *et al.* (25) checked some of the other previously reported cytophysiological observations on sieve elements. In mature sieve elements, they detected no tonoplast, or cytoplasmic streaming, or accumulation of neutral red. Like Bauer (4), they found no formazan in sieve elements in sections treated with tetrazolium.

Van Fleet (96, 97) reported presence of peroxidase in the phloem in all kinds of cells: sieve elements, companion cells, and surrounding parenchyma cells.

A deficiency in certain enzymes in sieve elements was deduced by Wanner

(103) from his analysis of phloem exudate of *Robinia pseudoacacia*. He obtained a positive test for phosphoglucomutase but for no other glycolytic enzymes. Several phosphatases were identified in the exudate but no invertase. Wanner suggested that the sieve-element protoplast loses most of its enzymes during maturation and is therefore not actively concerned with translocation.

Currier *et al.* (25) cautioned against conclusions about the degree of metabolic activity of sieve elements based upon such evidence as paucity of enzymes in the phloem exudate, or absence of cytoplasmic streaming, or failure of vital staining and of tetrazolium reduction. They pointed out, for example, that changes in the amount of callose on the sieve areas, which are particularly striking in species showing winter dormancy and spring reactivation of phloem [Esau (30)], occur in mature enucleate elements. It may well be that the peculiar structural characteristics of the mature sieve elements are associated with equally peculiar cytoplasmic activity.

The undoubted plasmolyzability of sieve elements weakens the concept of free movement of solution from element to element as required by the mass-flow hypothesis, but certain observations indicate that such movement is possible. Rouschal (78) observed a surging flow of liquid through a series of sieve elements of *Aesculus* in sections which were exposed to a glycerine solution at one end. The liquid flowed toward the glycerine, dislodged the carbohydrate granules and carried them toward the sieve plates. Rouschal interpreted the movement as mass flow and assumed that the protoplast-sieve plate complex is highly permeable. Similar observations were made by Bauer (4) on detached petioles of *Pelargonium*. Both cut ends of the petioles were submerged in solutions that were altered in composition according to the design of the experiment. Fluorescent dyes were introduced into the median part of the petiole through a shallow wound. As described by Bauer, his experiments utilized living material to illustrate Münch's (72) model system. According to Bauer, both the direction and the rate of movement of the dyes were under the control of artificially induced turgor gradients. Like Rouschal, he assumed a mass flow through connected series of sieve elements and a high degree of permeability at the sieve plates.

Free flow across the sieve plates also offers the best explanation for the well-known phenomenon of exudation from the phloem [Crafts (20, 21)]. The commonly observed displacement of cell contents in sieve elements upon cutting, which results in an accumulation of the so-called slime [cf. Esau (32)] and granules on the inner sides (those away from the cuts) of the sieve plates nearest to the cut ends of the sections, is best explained by the concept of mass flow of contents across the sieve plates. Currier *et al.* (25) observed a surging flow of contents within sieve elements during plasmolysis and deplasmolysis. They saw in this phenomenon an evidence of mass flow in the elements themselves.

Thus, since the physiology of phloem was last reviewed [Arisz (2)], additional evidence has been obtained to suggest that the sieve-element

XUM

protoplasts are in a less denatured state than is assumed by some proponents of the mass-flow hypothesis. On the other hand, there are also new data showing that under certain experimental conditions a rapid flow of liquid from element to element across the sieve areas can be demonstrated. Obviously, then, the sieve areas together with the protoplasm associated with them need renewed investigation.

Sieve Areas

The sieve elements may be best interpreted as specialized parenchyma cells and the sieve areas as modified primary pit fields. The differences between the sieve areas and the pit fields appear to be only quantitative. Both constitute thin areas in primary walls with clusters of protoplasmic strands penetrating the thin part of the wall. These strands are the plasmodesmata in the primary pit fields. In the sieve areas the strands are also often referred to as plasmodesmata, or sometimes as connecting strands [Esau (30, 32)]. Until recently the presence of callose on the sieve areas was emphasized as a distinguishing characteristic between the sieve areas and the primary pit fields in parenchyma cells. Now callose—or at least a substance showing the same staining reaction as callose—has been shown to be present in pit fields in a variety of cells [Currier & Strugger (26)].

Strands in sieve areas range in thickness from those comparable to plasmodesmata to strands that occupy, together with the callose enclosing them, pores of about 10 μ in diameter [e.g., *Robinia* and *Ulmus*, Volz (100)].

The question of continuity of plasmodesmata and sieve-area strands across the walls from protoplast to protoplast is raised from time to time. Recently Lambertz (69) concluded that plasmodesmata are, in general, highly labile structures that are withdrawn from walls under certain environmental conditions or in response to wounding. The picture that emerges is not of strands continuous from cell to cell but of tentacle-like extensions of the protoplast penetrating the wall. It is conceivable, of course, that plasmodesmata from two adjacent cells may come in contact with one another and thus perhaps establish continuity.

With regard to the continuity of the sieve-area strands, electron-microscopic studies by Huber & Kolbe (49) and Volz (100) indicate that the wide sieve-area pores are open; that is, the strands in these pores are continuous from cell to cell. Narrow pores, on the other hand, may be closed by a membrane so that the strands are interrupted in the median position. For example, in *Cucurbita* the pores in the sieve plate in the end wall are up to 10 μ in diameter and are open; the pores in the lateral sieve areas are about 0.1 μ in diameter and are closed [Volz (100)].

If the electron-microscope views reveal a true picture, one must assume that the continuity of the sieve-area strands is not a consistent feature and depends on the relative thickness of the strands. Currier *et al.* (25) have suggested that the sieve-tube members of the angiosperms, with their more or less highly differentiated sieve plates on the end walls, are more strongly

specialized with regard to longitudinal conduction than the sieve cells of the gymnosperms. If the increase in differentiation of the sieve areas were accompanied by the establishment of continuity of strands, the difference between the sieve-tube members of angiosperms and sieve cells of gymnosperms would be even more meaningful.

The composition of the sieve-area strands is most commonly compared with that of the plasmodesmata; that is, they are described as composed of cytoplasm [cf. Esau (32)]. The well-known divergent view that the strands have a tubular cytoplasmic sheath enclosing vacuolar material—such structure would obviously permit a flow of vacuolar contents from cell to cell—has recently been reported once more as untenable. According to Hepton *et al.* (44), in electron micrographs the connecting strands in *Cucurbita* sieve plates consist of cytoplasm. Esau (30), however, gave clear evidence that slime is present in the connecting strands in *Vitis*. It is somewhat questionable whether the present methods of preparation for electron-microscope studies would reveal the distinction between cytoplasm and slime.

Lack of continuity in the finer strands and absence of vacuolar communcation through the wider strands would make it difficult to visualize a free flow across the sieve areas unless the cytoplasm here had some peculiar properties. Rouschal (78) has suggested that the sieve-area strands may consist of plasmalemma enclosing some inner cytoplasm. A continuous strand would thus have no plasmalemma separating the protoplasts of two adjacent cells. The solution would still have to move across the tonoplast if present. Rouschal implies that a tonoplast, even if it were normally developed in sieve elements, would be less restrictive than the plasmalemma.

The new information on connecting strands just reviewed and that on plasmolyzability of mature sieve elements are not the only factors demanding a renewed study of sieve areas. Reliable information recently obtained clearly shows that the development of sieve elements frequently involves divisions in cambial derivatives, either transverse or longitudinal or both, that result in shorter and narrower cells and a larger number of end walls (site of future sieve plates) than would be expected from a study of the shape and length of cambial initials [Esau & Cheadle (33)]. Thus, the ontogenetic sequence may be such that the potential size of the conduit is specifically reduced and the number of sieve plates increased. The proponents of the view that movement in the phloem occurs in the cytoplasm, especially along its surfaces, would regard this development not as impeding but as definitely expediting movement. Schumacher (81) has reported that, whereas the lumen of the conduit was much reduced in the sieve-plate pores, the cytoplasmic surface was increased two to four times.

Attempting to reconcile the cytoplasmic nature of the connecting strands with the mass-flow concept, Crafts (23) visualized a straightening and ordering of the peptide chains accompanied by a loosening of bonds and loss of substances responsible for differential permeability. Water, mostly in free state, would replace these substances. According to Crafts, such a skeleton

XUM

of peptide chains holding a large quantity of water, and agitated thermally, would offer little resistance to the intercellular flow of solution.

Our lack of understanding of the role of connecting strands in translocation is matched by our ignorance regarding the significance of callose development in the sieve areas. As mentioned, the recent studies by Currier & Strugger (26) showed that callose can be associated with ordinary plasmodesmata in parenchyma cells. In the sieve elements callose development is more pronounced and, as is well known, relatively large accumulations of the substance occur at the time of reduced or discontinued activity in sieve elements [cf. Esau (32)]. Injury also induces a deposition of masses of callose on the sieve areas [Schumacher (82)].

The chemical composition of the callose has been variously interpreted. One of the old views that the callose is proteinaceous in nature and is related to the slime has been again revived [Salmon (79)]. Küster (68), on the other hand, concluded that it was related to cell-wall substances. In contrast to other workers, he reported that the callose of *Cucurbita* was soluble in cuprammonia and was anisotropic. Eschrich (36) found that the substance in cystoliths of *Ficus*, which he identified as callose by the resorcin-blue staining test, gave D-glucose upon hydrolysis with hot H_2SO_4.

Parenchyma Cells in the Phloem

The peculiar structure of the sieve-element protoplast, particularly its enucleate condition, suggests that associated parenchyma cells may play an important role in translocation, perhaps by reactions mediated by enzymes present in these cells and absent in the sieve elements. Conceivably such reactions may bring about secretion into or absorption from the conduit, the sieve element.

Some recent studies suggest that certain parenchyma cells associated with the phloem are particularly concerned with the transfer of carbohydrate assimilates from the mesophyll cells to the sieve elements. Using *Robinia* and *Carpinus*, Wanner (102) identified sugar phosphates in the mesophyll and found sucrose but no hexoses or sugar phosphates in the phloem exudate. This observation, together with the data indicating intense phosphatase activity in companion cells and border parenchyma (vein sheath) cells [Frey (38)], led Wanner (101, 102) to suggest that the parenchymatous cells split off phosphate and synthesize sucrose which is in turn, or at the same time, secreted into the sieve element. Thus, the sieve element would accumulate sucrose rather than hexoses. Phosphatase activity would insure a gradient of decreasing concentration of sugar phosphates from the mesophyll to the parenchyma associated with the phloem. Bauer (4) obtained similar results on phosphatase distribution, but concluded that the main activity of such enzymes occurs within the phloem itself (companion cells and transitional cells at bundle ends) rather than in the border-parenchyma cells. He developed further the concept of movement of assimilates by suggesting

that secretion of sucrose into the sieve elements attracts water osmotically, a process which creates a high turgor pressure. Thus the turgor mechanism is located in the phloem of the fine veins and is created by the activity of the parenchyma cells in these veins.

The mechanism proposed by Wanner and Bauer would help to explain the movement of assimilates from the mesophyll to the sieve elements despite a lower osmotic pressure in the mesophyll [Bauer (4); Roeckl (75); Wanner (101)].

The considerations just presented indicate one of the possibilities of active participation of parenchyma cells in translocation. One may assume that many others exist. Morphologic studies suggest that parenchyma cells are constant members of the phloem tissue and some are apparently more highly specialized than others in regard to the activities of sieve elements. Such specialization has not yet been recognized in the lower vascular plants [cf. Esau *et al.* (34)]. In the gymnosperms the so-called albuminous cells, which occur in the rays and in the vertical system of the secondary phloem, are structurally and functionally connected with the sieve cells and the usual absence of starch indicates some specialization of their protoplasts. The highest degree of specialization in sieve element-parenchyma relation is apparently attained in the angiosperms in which the companion cells originate as sister cells of the sieve elements. Like the albuminous cells of the gymnosperms the companion cells of angiosperms accumulate no starch and ultimately become functionless simultaneously with the associated sieve elements [Esau *et al.* (34)].

The specific relation between companion cell and sieve element has been much emphasized in the literature and the view commonly expressed that the nuclei of the companion cells control the activities of the enucleate cytoplasm of the sieve elements. Recent studies suggest that companion cells intergrade with parenchyma cells in degree of association with the sieve elements. Holdheide (45) and Huber & Graf (48) reported that in the secondary phloem of many woody dicotyledons parenchyma cells with relatively dense cytoplasm and large nuclei occur next to sieve elements and collapse when the latter cease to function. According to Resch (74), in the primary phloem of *Vicia faba* the sister cells of sieve elements vary in degree of development of characteristics usually associated with companion cells. Esau & Cheadle (33) made similar observations on secondary phloem of woody dicotyledons. Parenchyma cells derived from the same mother cell as the sieve element may be more or less distinct cytologically from the companion cells; and these parenchyma cells may collapse when the sieve elements cease to function while other parenchyma cells remain alive and continue to store starch.

Thus, one might suggest that companion cells intergrade with phloem parenchyma cells in degree of physiologic specialization, whatever this specialization may be. Furthermore, the phloem-parenchyma cells that are

XUM

not closely associated with the sieve elements also show visible differentiation with regard to shape, wall structure, and contents. The subject of parenchyma in the phloem is obviously in need of further exploration.

Studies on Movement in the Phloem

Movement of fluorescent dyes.—Studies on movement of fluorescent dyes are of special interest because the location of the dye in the particular cells of a tissue can be visually determined. Long-distance movement of fluorescent dyes has been seen to occur in the sieve tubes and their companion cells [Eschrich (35); Bauer (3, 4); Schumacher (81); Schumacher (83, 84)]. Indeed, of all the substances whose translocation in plants has been studied, the fluorescent dyes are the only ones whose movement was definitely associated with the sieve elements. However, in view of the inconsistencies in the results of studies on translocation of various materials [cf. Arisz (2)], there is no certainty that the movement of the dye duplicates that of the assimilates.

Despite the visibility of the dye in the tissue and the use of the same dyes and the same plants, studies involving fluorescent dyes have led to two opposite conclusions regarding their movement in the phloem: first, it is an accelerated molecular movement occurring in the cytoplasm of the sieve elements [Schumacher (84, 86, 87); Schumacher (81)]; second, it is a passive movement in a streaming solution that occurs through the vacuoles [Rouschal (78); Bauer (3)]. Schumacher (84, 86) saw proof for molecular movement especially in two experimental observations. (*a*) A small amount of dye would move for a limited time and then stop. Addition of the same dye would force the front to advance farther. (*b*) This "pushing" effect could be obtained only with the same dye. Different dyes moved independently. The proponents of the mass-flow hypothesis advance principally the two following arguments in favor of their view: (*a*) the dye is visible in the vacuoles and (*b*) the movement is affected by osmotic conditions.

Since the last reviews on translocation were written [Arisz (2); Crafts (24)], detailed studies on movement of fluorescent dyes were carried out by Bauer (4). From preliminary studies on parenchyma cells and plant hairs Bauer concluded that the main movement of uranin (Na-fluorescein) occurs through the vacuoles, although optically it may be visible only in the cytoplasm. He explained this optical effect as resulting from an accumulation of the dye in the cytoplasm. In contrast, rhodamine-B may be seen in both vacuole and cytoplasm. Bauer emphasized the observation that a suction-pressure (DPD), created by letting the hairs partially wilt, caused an acceleration of the movement of the dye. He interpreted such acceleration as proof of movement in the vacuoles.

With regard to movement in the sieve tubes, Bauer proposed to determine the mechanics of movement and the relation of dye movement to that of the assimilates by studies involving experimentally induced changes in the movement of dye. Such changes should be reflected in (*a*) the rate of

movement and (*b*) the direction of movement. To make the determinations as precise as possible Bauer used detached leaves or debladed petioles—detachment from the plant removes the possible accelerating effect of places of utilization or storage of the assimilates—and selected his material for uniformity in age and size. Furthermore, he restricted his studies to the large central vascular bundle characteristic of *Pelargonium* petioles. The dye was applied in gelatin through an incision in the petiole, 1 cm. below the blade, or in the middle of the petiole if the blade was removed. The number of experiments was so large that the data were analyzed statistically.

As the basic rate of movement Bauer used the 12 to 16 mm. per hr. rates obtained in turgid leaves just removed from potted greenhouse plants and kept, after the introduction of the dye, in a darkened moist chamber for three hours. He pointed out that these rates were considerably lower than the 20 to 500 mm. per hr. observed by Schumacher (83, 86) in undetached leaves of *Pelargonium*, but were sufficiently high to rule out movement by simple diffusion. He studied the effect of the following factors upon the rate of movement: (*a*) presence of blade, (*b*) degree of water saturation of leaves, (*c*) respiration, (*d*) temperature, (*e*) length of storage of detached leaves, (*f*) presence of light, (*g*) feeding with sugar, (*h*) treatment with phloridzin, a phosphorylation inhibitor.

Bauer found that the movement of the dye in the petiole was retarded by removal of the blade and by reduction of degree of saturation of the leaf with water. Lowering of temperature at the blade had a stronger effect upon movement than cooling of the petiole. Similar differences were found in the effect of inhibited respiration. Bauer saw in these responses an evidence that the driving force (motor) of the translocation resided mainly in the blade, specifically in the bundle ends where sucrose may be secreted into the sieve elements by the phosphatase-containing parenchyma cells.

Various other experiments suggested to Bauer that the movement of dyes was directly dependent upon the simultaneous movement of organic materials. This view was additionally supported by the observation that in intact variegated plants the dye always moved toward the wholly white leaves or white areas on mottled leaves.

For studies on the direction of movement of the dye, Bauer (4) used the previously mentioned experimental design, a petiole with both ends inserted in vials and the dye applied through a wound in the median position. By varying the contents of the vials, so as to induce either small or large DPD effects at one or the other end of the petiole, he was able to change the rates of movement as well as completely to reverse the direction of the movement.

According to Bauer (4), the mass-flow hypothesis gives the best explanation of the results of his experiments. Schumacher & Hülsbruch (88), however, do not agree that conclusions about a mechanism in an intact plant are necessarily valid when drawn from information obtained by means of severed plant parts. Furthermore, they criticize Bauer for using relatively small pieces of plant and studying movement of dyes over very short dis-

XUM

tances and at very low rates. They also point out that Bauer has not suggested how the dye moving in the vacuoles crosses the cytoplasm of the sieve plates.

A recent study on movement of fluorescein by Eschrich (35) was concerned with re-establishment of phloem connection after severance of one of the vascular bundles in the stem of *Impatiens*. The dye introduced into the severed bundle moved out of the bundle into the parenchyma at the edge of the wound, then proceeded sideways toward the intact bundles. In these bundles, new sieve tubes differentiated in continuity with the wound sieve tubes at the ends of the severed bundle. The dye from the severed bundle then entered the wound sieve tubes and their continuations in the intact bundles. After the ends of the severed bundle became connected through regeneration of sieve tubes, however, the dye once again became restricted to the sieve elements and their companion cells.

Eschrich emphasized that a weak conduction of fluorescein began in incompletely differentiated elements and that it occurred at the usual rates after the sieve plates had differentiated but before the nuclei had broken down. He cautioned against the notion that certain structural features must be present before a particular function occurs.

The problem of movement of fluorescent dyes is obviously complex. In view of the careful techniques applied by both Schumacher and Bauer, one is forced to assume that the divergencies in the basic conclusions resulted from differences in the experimental approach and in the interpretation of observations.

Movement of viruses.—The information on the movement of viruses and on the relation of this movement to the translocation of assimilates is impressive in its scope and convincingness [cf. Bennett (6, 7); Crafts (22, 24); Esau (31)]. Evidence that the systemic invasion of plants by viruses occurs through the phloem has been obtained not only for the phloem-limited viruses (yellows type, e.g. curly-top virus), but also for those that readily invade parenchyma (mosaic type). Infection of plants with phloem-limited viruses depends on the initial introduction of these viruses—usually by means of insect vectors—into the phloem. The mosaic viruses are readily introduced mechanically into the epidermis or parenchyma, but move slowly in these tissues. When they reach the vascular tissue, their movement is suddenly accelerated.

The principal evidence of movement of viruses in the phloem was obtained by (*a*) ringing experiments and (*b*) experiments designed to influence carbohydrate movement in virus-infected plants [cf. Bennett (6)]. The spread of viruses is interrupted or delayed when the phloem is removed from their path by ringing. Most of the studies involving carbohydrate translocation in virus-infected plants indicate a close agreement between the direction of movement of viruses and that of the carbohydrates [Bennett (7)]. Moreover, the rates of movement of viruses in the phloem [cf. Bennett (6)] are

similar to those obtained for the movement of food and radioactive substances [cf. Biddulph (9)].

The experimental details of the research on translocation of viruses and the discussion of the possible mechanism of this translocation have just been reviewed by Bennett (7) in this publication. The principal reason for including a review of virus movement in the present paper is to add to Bennett's summary the pertinent information from a recent paper by Zech (104). Using tobacco mosaic, Zech confirmed certain earlier observations that upon inoculation into a leaf of *Nicotiana tabacum* the virus moves slowly at first, then more rapidly after a vein area is reached. In contrast to earlier interpretations [Holmes (46, 47)], however, Zech suggested that the initial acceleration occurs in the vein parenchyma (bundle sheath, for example) rather than in the vascular bundles themselves. He ascribed considerable importance to the length of cells and assumed that the elongated shape of the vein parenchyma cells is causally related to the speed of virus movement along the veins. Zech (104) recognized cytological abnormalities long before the appearance of inclusion bodies. Using this early symptom as a guide he calculated the following rates of movement of the virus: 0.18 mm. per hr. in epidermis between the veins; 0.4 mm. per hr. in elongated epidermal cells along the veins; 8 mm. per hr. in elongated bundle sheath cells.

From the inoculated leaf the virus moved rapidly through the stem, mainly to the root. The rate of this movement was not calculated but Zech agreed with the usual concept that this rapid transport of the virus toward the root occurred in the phloem, especially in the sieve tubes. He stressed, however, that the sieve tubes themselves did not show any abnormalities, even when the associated parenchyma cells contained the typical mosaic inclusions in abundance. Similar observations were previously made by Esau (29).

Köhler (55), using another measuring device, also related differences in length of parenchyma cells to the different rates of movement of two potato viruses. When, for example, the X virus was inoculated over the main lateral veins where the cells are elongated the rate was 0.083 mm. per hr., whereas when the inoculation was over the intercostal area the rate was only 0.036. Excluding a possible incubation period of 24 hours the maximum rate would be 0.125 mm. per hr. Köhler also studied the rate of movement in the stem. He obtained the values of 0.6 to 0.7 cm. per hr. for the Y virus and 0.2 cm. per hr. for the X virus. He suggested that the slower movement of the X virus resulted from its inactivation in the phloem but that both viruses moved in the phloem within the stem. This suggestion, Zech's conclusion on the transport of precursory particles (see below), and the common observation that incubation periods precede the rapid movement through the plant, all emphasize the inherent difficulties in determining rates of transport of viruses.

Zech (104) paid considerable attention to the discontinuous distribution

XUM

of infection during the initial systemic movement of the virus from the inoculated leaf toward the root. Such distribution was noted repeatedly in studies with both yellows type [Bennett (5)] and mosaic viruses [Capoor (15); Köhler (54); Kunkel (56); Samuel (80)]. Most of the previous workers interpreted this phenomenon as proof of mass flow of virus in the phloem. The translocation stream carries the virus passively along and, since at this first stage of systemic infection the amount of virus is still low, parts of the phloem may fail to retain any virus particles. Zech, however, developed the concept that the virus is transported not in the form of complete molecules, but as smaller noninfective precursory particles. As some workers before him, Zech considered that complete virus particles were much too large to pass from cell to cell even if they were moving through plasmodesmata. Zech explained the discontinuous spread of virus by assuming that the noninfective precursory particles were incapable of combining into the complete virus particles within the enucleate sieve elements. To combine they must move into parenchyma cells. During the rapid long-distance transport, however, the particles do not leave the sieve elements in intermediate locations, although they may do so later. If this interpretation is correct, then one must assume also that the precursory particles may remain confined to the sieve elements indefinitely, since in Bennett's (5) and Kunkel's (56) experiments the apparently virus-free stem pieces were rooted and grew into flowering plants without showing any symptoms.

If the movement of viruses in the sieve element does not occur according to Zech's concept, the question must be asked, whether the large virus molecules are capable of entering the sieve elements from the adjacent parenchyma cells if they are not introduced directly into the phloem by insects. In the sieve elements themselves the connecting strands of the sieve plates are large enough to permit passage of these large molecules. The movement of viruses in the sieve elements is merely part of the general problem of translocation of materials in the phloem.

Translocation in nectaries.—A series of studies on nectaries has revealed a close relation between the vasculature of nectaries and the composition of nectar. These studies provide evidence for the movement of sugar in the phloem. Floral nectaries of the dicotyledons [Frei (37); Frey-Wyssling (39)] may have a special vascular system beneath the glandular tissue or they may lack this system and thus obtain food and water from the nearest floral bundle. The special vascular system usually contains phloem tissue only or a combination of xylem and phloem in which the phloem predominates. In the floral nectaries of *Fritillaria*, however, xylem predominates in the vasculature [Agthe (1)]. The extra-floral nectaries show considerable variation in the amounts of xylem and phloem in their vascular system. In fact, they intergrade with the floral nectaries supplied by phloem on the one hand and the hydathodes supplied by xylem on the other [Agthe (1); Frey-Wyssling & Agthe (40)].

Studies on the amount of sugar in the various kinds of nectaries have

shown that nectaries supplied by phloem have a high sugar concentration; those containing both xylem and phloem produce a noticeably more dilute nectar. For example, the nectaries of *Euphorbia* and *Abutilon*, which are rich in phloem, may contain 50 to 60 per cent of sugar in the nectar. A much lower per cent of sugar occurs in the phloem and xylem-containing floral nectaries of *Ranunculus* and *Fritillaria* and foliar nectaries of *Ricinus*. As mentioned, xylem predominates in the floral nectaries of *Fritillaria* and their nectar contains only about 8 per cent of sugar [Agthe (1)].

This relation between the structure of the vasculature of the nectary and the composition of nectar obviously points to phloic origin of the sugar in the nectar. Experiments with nectaries fed with sugar point in the same direction: placement of inflorescences, flowers, or nectaries in sugar solutions induces continuous sugar excretion by the nectaries, with a close correlation between the sugar content of the solution and that of the nectar [Zimmerman (105)]. The total output of nectar also appears to be increased by feeding with sugar [Agthe (1); Pankratova (73)].

In some experiments with fluorescein [Agthe (1)], the dye entered nectaries after being placed at a considerable distance from the nectary. However, the dye moved only into actively excreting nectaries. This observation was interpreted as indicating movement of fluorescein with the sugar. Fluorescein was not excreted, however, and Agthe suggested that the dye was stored in the cytoplasm.

The relation between vasculature and activity of nectaries graphically illustrates that carbohydrates move in the phloem, and the illustration is based on intact plants. It is pertinent to emphasize in this connection that the phloem supply to the nectaries always contains sieve elements [Frei (37)]. In other words, differentiated phloem occurs in the nectaries, not "undifferentiated conducting strands," as is often reported in the literature.

Movement as revealed by radioactive tracers.—In a recent review on translocation of radioactive substances, Biddulph (10) expressed disappointment that studies involving these substances did not yield the expected results. He suggested that the cause of the difficulty may be the lack of suitable methods of introducing the substances into the phloem. One of the common techniques of introducing the solution into the plant through a leaf does not assure entry into the phloem exclusively.

What is the evidence that the radioactive substances used by various workers are actually moving in the phloem? Studies designed to determine the pathway of radioactive substances involved a technique of partially stripping the bark so that the bark and the wood could be checked separately for the presence of these substances. Using this technique, Stout & Hoagland (89) showed that radioactive isotopes of potassium, sodium, phosphorus, and bromine introduced into roots move upward through the xylem tissue. Biddulph & Markle (11) introduced P^{32} into a leaf and found that it moved downward in the bark. Chen (16) observed upward movement of radiophosphorus mainly in the xylem, and downward movement from the leaf

XUM

mainly in the phloem. When $C^{14}O^2$ was introduced into a leaf C^{14}-labeled photosynthate moved in the phloem. In experiments with radioactive phosphorus Colwell (17) found high concentration of radioactive material in the phloem exudate of *Cucurbita*. By refining the autoradiographic technique Biddulph (12) obtained visual evidence that S^{35} and P^{32}, introduced into a leaf, are translocated principally in the phloem. The experiments of Thaine & Walters (93) with autoradiographic techniques have not yet yielded conclusive results regarding the pathway of isotopes.

Radioactive substances are particularly suitable for the study of rates of movement in the phloem. In Russian studies with isotopes [Kursanov (60)] rates of 30 to 100 cm. per hr. were determined for products of photosynthesis moving from leaves to roots in various plants. The rate was found to vary in relation to the stage of development of the plant and also to the direction of the movement. According to Vernon & Aronoff (99), sucrose was translocated at the rate of 84 cm. per hr. in soybean stems. Biddulph (8) reported rates of movement ranging between 17 and 88 cm. per hr. for radiophosphorus in the phloem of the bean plant.

The use of radioactive substances has demonstrated that there may be simultaneous movement in two directions, upward and downward [Biddulph & Markle (11); Chen (16)]. This observation is considered as evidence against the mass-flow hypothesis. Actually, this hypothesis does not require a unidirectional movement within the entire phloem system of leaf, stem, or root. If a given substance is introduced into a leaf and then, upon reaching the stem, is diverted upward and downward [Biddulph & Markle (11)], or if two substances are introduced into two different leaves and move in different directions [Chen (16)], the movement in opposite directions need not necessarily occur in the same phloem strand.

Simultaneous bidirectional movement in the same vascular bundle has been observed, however, in experiments with fluorescent dyes. Bauer (4) found that in certain experiments, designed to reverse the direction of movement of fluorescein in the central vascular bundle of a *Pelargonium* petiole, the dye moved simultaneously in two directions. According to Bauer (4, p. 417), usually it was possible to recognize that the movement in the two directions occurred in different sieve tubes. In his reversal experiments with *Cucurbita*, Rouschal (78) observed that, when fluorescein was translocated in two directions simultaneously, the movement in external and internal phloem occurred in opposite directions.

Use of radioisotopes has shown that different substances may move at different rates—another observation that is used as evidence against mass flow. Vernon & Aronoff (99) supplied $C^{14}O_2$ to soybean plants and reported that the resulting labeled sucrose had the highest rate of translocation, followed by glucose and fructose in the order given. Swanson & Whitney (91), applying P^{32} and K^{42} or P^{32} and Cs^{137} simultaneously to leaves, found independent and different rates of movement for the different isotopes. According to Biddulph (8), phosphorus and sulfur move freely in the phloem,

XUM

whereas iron, calcium, and zinc do not. Biddulph (8) also thinks he has furnished evidence that the movements of P^{32} and tritiated water are not closely correlated [cf. also van Overbeek (98)].

Relation of movement to metabolism.—The proponents of the different theories of translocation visualize the participation of the living cells in the movement of materials in the phloem. Crafts (e.g. 18, 19, 24) has repeatedly stressed the importance of active processes in parenchyma cells with reference to the translocation mechanism. He suggests, for example, that storage-parenchyma cells can induce a lowering of concentration in the phloem by accumulating sugar and thus be instrumental in the polar transport of assimilates. The "sink," in other words, is provided by the living cells, storing or actively growing and dividing. The recently postulated method of delivery of sugar against the concentration gradient into sieve elements of small veins in leaves by phenomena of phosphorylation and dephosphorylation also assumes participation of parenchyma cells in translocation. (See part on parenchyma cells.)

Information on the effect of temperature on translocation has a bearing on the question of relation between metabolism and translocation. The older literature dealing with this effect has presented two opposing views [cf. Crafts (24)]. Observations that the content of carbohydrates in tomato plants decreased when temperature was elevated led to the conclusion that the rate of translocation was reduced at the higher temperatures. The contrasting view is that the decrease in carbohydrate reserves at higher temperatures may result from speeded up respiration and that, up to a certain optimum, translocation is actually more rapid at these higher temperatures.

Recently Hull (50), after having followed the movement of C^{14}-labeled glucose or sucrose in sugar beet and tomato plants at different temperatures, concluded that translocation was as rapid or more rapid at lower temperatures. In contrast, a series of closely integrated papers reported an acceleration of translocation by increase in temperature. Swanson & Böhning (90) immersed the blade end of one leaf of a bean plant in a sucrose solution and observed the effect of cooling or warming of the petiole of this leaf upon the growth of the plant held in the dark at $20 \pm 1°C$. The translocation was retarded by cooling and speeded up by warming of the petiole. Similar effects of temperature upon translocation from bean leaves were obtained by cooling the hypocotyl below the experimental leaves [Böhning *et al.* (14)]. Intermittent low and medium cooling of the petiole of a bean leaf had a more pronounced retarding effect upon translocation than continuous cooling, whereas intermittent medium and high temperature had a less pronounced accelerating effect than continuous warming [Kendall (52)]. In tomato plants also, low temperatures were found to have a depressing effect on the movement of carbohydrates [Böhning *et al.* (13)]. Inhibition of translocation by low temperatures was confirmed by the use of radioisotopes for the kidney bean [Swanson & Whitney (91)] and for the soybean [Vernon & Aronoff (99)].

The effect of temperature on the movement of fluorescein determined

XUM

by Bauer (4) was reviewed earlier in this paper. Bauer extended his studies on the effect of temperature by determining the elongation and the dry weight of adventitious roots of *Helianthus annuus* seedlings whose hypocotyls were exposed to different temperatures. The taproot was cut off in seedlings bearing the first pair of foliage leaves and the formation of adventitious roots induced by appropriate cultural means. The plants were selected for uniformity and the roots trimmed down to a certain number. Lowering of temperature did not affect the further elongation of the roots but it reduced their dry weight.

Thus the majority of studies on effect of temperature show that movement of sugars, radioisotopes, and dyes is retarded when temperatures are lowered below a certain optimum. Two explanations of this temperature effect are possible, although they are not necessarily mutually exclusive. They are: (*a*) an increased viscosity of protoplasm and cell sap, and (*b*) a direct effect on respiratory reactions supplying energy for the process of transport. Bauer (4), for example, concluded that the predominant effect of low temperature in the blade of *Pelargonium* is respiratory inhibition, while the effect on the petiole is mainly an increased viscosity. This conclusion conforms with his finding that with respect to transport the sieve tubes of the petiole are relatively insensitive to metabolic inhibitors. There is not enough published information, however, to establish a conclusive explanation of the exact nature of the temperature effect.

One of the methods used to determine the relationship of metabolism to phloem translocation is the use of inhibitors. In some instances decreased rates of transport were observed. Bauer (4) applied phloridzin, a reagent affecting phosphate transfers, to the *Pelargonium* leaf blade and noted a lowered rate of downward transport of uranin in the petiole; but no inhibition resulted when the petiole itself was treated with the reagent. Kendall (53) injected inhibitor solutions into the hollow petioles of primary bean leaves. Movement into the stem of P^{35} applied to the blade was inhibited by dinitrophenol (DNP) and by sodium fluoride (NaF), but not by 2,4-D, indoleacetic acid (IAA), and triiodobenzoic acid (TIBA). Sodium arsenite gave inconclusive results. Like phloridzin, DNP and NaF would be expected to interfere with phosphate transfers [James (51)]. The effectiveness of petiole-applied inhibitors reported by Kendall contrasts with the negative results obtained by Bauer with phloridzin and by Vernon & Aronoff (99) with HCN.

Turkina & Dubinina (94) reported that the respiration of vascular bundles isolated from leaves was strongly depressed by KCN but less so by diethyldithiocarbamate and concluded that the conducting tissues had a much more active cytochrome-oxidase system than other living tissues.

The depression produced by cyanide would indicate dependence of transport on oxygen. The effect should be similar to the inhibition produced by a nitrogen environment [Bauer (4)]. The inhibition of dye movement in the petiole by the mere infiltration of the leaf blade with tap water demonstrates the sensitivity of the system to oxygen.

XUM

Hay (43) reported that TIBA inhibits longitudinal translocation of IAA in isolated 5-mm. segments of bean stem and petiole. In these experiments phloem may not have been involved specifically. Diffusion and protoplasmic streaming in parenchyma cells might well have accounted for the movement. Inhibition of transport of asparagine and phosphate in specialized parenchyma cells (tentacle cells of *Drosera*) by several diverse metabolic inhibitors has been reported by Arisz (2).

At present we may only speculate as to what system is affected by the respective inhibitors. In addition to effects on those active processes within the parenchyma cells contributing to the transport in phloem and inhibition of "activated diffusion" in the sieve-element protoplasm, two other possibilities present themselves: a reduced permeability of the cytoplasm of the sieve areas and an increased callose accumulation with the resulting constriction of the connecting strands. It should be remembered, however, that it is not always certain, as was pointed out by Vernon & Aronoff (99), that the inhibitor penetrates to the phloem; this lack of certainty would seem to be more of a problem with regard to the petiole than the blade.

Although the dependence of translocation on the activity of living cells is generally recognized, the mechanism of transport in the sieve elements is usually discussed and analyzed as a physical phenomenon. The Russian physiologists criticize this approach and describe numerous experiments, dealing with various aspects of the physiology of conducting tissues, that are thought to furnish proof of an interdependence between movement of food materials and metabolic activity [Kursanov (58, 60, 61)]. The movement is characterized as a process of "pushing through," whereby the substance moves simultaneously along the entire conducting system without a disturbance of the steadiness and continuity of the concentration gradient within this system. The support of this particular concept is based on studies of movement of sugar in sugar-beet leaves during the period of most active accumulation of sugar in the root [Kursanov *et al.* (65)]. Vascular bundles stripped from midveins and petioles showed very little fluctuation in the amount and relative composition of sugar during a 24-hour period. At the same time, in the blade, the amount of sugar underwent considerable changes: it moved out of the blade in the first—light—part of the 24-hour period, then again accumulated between 7 P.M. and 7A.M., that is, during the dark period. The conclusion was drawn that during the 24-hour period sugars moved first in one then in another direction without noticeable changes in concentration of sugars in the conducting tissues.

The occurrence of actual transport of sugars in the vascular bundles was revealed by infiltrating blades with a 1 per cent solution of sucrose labeled with C^{14} and following the movement of the radioactive isotope [Kursanov *et al.* (65)]. Fifteen minutes after the infiltration the isotope was present at the base of the petiole. Since the petioles were approximately 18 cm. long, the rate of movement was 70 to 80 cm. per hr. The isotope was found only in the isolated vascular bundles, none in the parenchyma of the petiole. The authors concluded that sugars move rapidly through the vascular bundles

XUM

without changing the general level of sugars, a conclusion used as argument against the movement as a mass flow or as a purely diffusional phenomenon.

The report by Kursanov and co-workers on unchanging level of sugar in sugar-beet leaves is difficult to evaluate. Numerically, parenchyma cells predominate in the vascular bundles of leaves (phloem parenchyma and xylem parenchyma) and the sugar content in these cells can be expected to overshadow that in the sieve elements. In other words, analyses of entire bundles give no indication of the sugar content in sieve elements, and not even in the phloem tissue.

Chemical analyses of conducting tissues and studies of distribution of C^{14}, supplied in carbon dioxide or in labeled sugars, showed sucrose to be the dominant sugar [Kursanov *et al.* (65); Kursanov & Turkina (64)]. The same evidence was obtained in experiments involving introduction of fructose into leaves [cf. Kursanov (60)]. When cut sugar-beet leaves were placed in a solution of labeled fructose, the major part of radioactivity in the vascular bundles was found in sucrose. When the latter was hydrolyzed, the products, glucose and fructose, were both radioactive in comparable degree. Krusanov concluded that the moving fructose underwent phosphorylation and isomerzation, and the resulting glucose phosphate ester reacted with the "phosphorylated fructose" so completely that no free radioactive glucose remained.

Since sucrose is not an electrolyte, the Russian authors consider that this sugar cannot move according to the molecular mechanism envisaged by them. They reported finding considerable amounts of phosphorylase and, particularly, of phosphatase in the isolated vascular bundles and suggested that the moving form of sugar could be a phosphate ester, whereas the sucrose remains stationary and serves as a source for the moving component. A possibility of the coupling with boric acid to form an ionizable sugar-boric complex [Gauch & Dugger (41)] was later suggested also [Kursanov (60)]. Wanner's failure to find sugar phosphates in the phloem exudate of *Carpinus* and *Robinia* (102) does not suggest that phosphorylated sugars are translocated in the sieve elements.

A generally high enzymatic activity in conducting tissues was reported by Kuprevich (57). Tests were made with woody dicotyledons and gymnosperms. Shavings of wood, stem pieces with bark removed, and pieces of bark, placed into various media containing the substances to be digested, were supposed to show enzymatic activity in xylem, cambium, and phloem, respectively. While this method might be satisfactory for testing the enzymatic activity in the xylem, stem pieces with bark removed cannot be considered as representing cambium. With regard to phloem, pieces of bark obviously would contain besides the active phloem one or more other tissues —such as cortex, parenchyma of the nonconducting part of the phloem, possibly phelloderm—so that a test with bark tells very little, if anything, about the enzymes in the conducting part of the phloem; most importantly, such a test gives no specific information whatsoever on the enzymes in the sieve elements themselves.

XUM

It may be recalled here that Wanner (103) found the phloem exudate of *Robinia* to be deficient in enzymes. Frey (38), on the other hand, reported that phosphatase activity could be detected in sieve elements, although he was not entirely certain whether the lead phosphate was formed in the sieve elements or diffused there from the companion cells. He also pictured lead-phosphate accumulation on a sieve plate but suggested that such localization might have been an artifact. It must be emphasized, however, that Frey found phosphatase activity in various kinds of tissues and reported that in some plants the xylem showed stronger activity than the phloem.

The reportedly high rate of respiration of conducting tissues is given as further evidence that translocation is metabolically activated [Kursanov & Turkina (62, 63)]. In studies on the comparative rates of respiration of vascular tissues and mesophyll, measured by oxygen uptake, the vascular bundles were stripped from leaves of *Plantago major* and the sugar beet. The respiration of the isolated bundles was found to be several times more intense than that of the parenchyma of the petiole. It even exceeded the respiration of the "mesophyll without veins"—presumably mesophyll with the major veins stripped out, but with the extensive minor venation system still remaining. No data are given on respiration of leaf tissues that were not subjected to the extrication of the vascular bundles. Thus, information on a real control excluding the effect of injury upon the tissues is not available. Furthermore, no sufficient details on methods were given to estimate the possible influence of the presence of cuticle on the pieces of mesophyll and petiole parenchyma as contrasted with its complete absence on the isolated vascular bundles.

In calculating the rate of respiration of phloem on the basis of its transectional area in the vascular bundles the authors excluded the xylem from consideration because, they stated, most of it had no living contents; and they regarded the collenchymatous bundle sheath cells as metabolically insignificant because of scarcity of cytoplasm. Assuming then that phloem mainly was responsible for the intense respiration of a given sample of a vascular bundle, this tissue was estimated to be consuming about 5000 μl. O_2 per hr. per gm. of fresh weight. This figure is, of course, of doubtful value because the authors eliminated from their calculations a high proportion of other living cells, the xylem parenchyma cells.

From determinations of the CO_2/O_2 ratios it was concluded that materials respired were carbohydrates [Turkina & Dubinina (94)]. The oxygen necessary for the respiration of the vascular tissues is derived, according to Kursanov (59), from the photosynthetic activity of the chlorophyll-containing cells that accompany the vascular tissue. Although such association is common while the stem is young, later the outer phloem becomes inactive and the new conducting phloem is usually removed from chlorphyll-containing cells. The root phloem, too, is not associated with chlorenchyma.

Treatment of isolated vascular bundles of *Plantago* and sugar beet with sucrose was found to increase their respiration [Kursanov & Turkina (63)]. With regard to *Plantago*, sucrose had the same effect whether the 5 cm.-long

XUM

pieces of bundles were submerged in the sugar solution in the respirometer flask or brought in contact with it by inserting one-fourth of the piece into the solution. In other words, treatment of a short length of a bundle showed the same total increase as submergence of the entire piece. This observation seemed to contradict the results of another experiment that showed a correlation between the increase in respiration induced by sucrose and the amount of tissue placed in the sugar solution. The authors concluded that in the suspended bundles the effect of sucrose spread from the submerged end throughout the piece, "probably because of movement of sucrose in conducting tissues." Moreover, in completely submerged bundles the presence of bundle sheaths in *Plantago* was assumed to retard the entry of sugar except at the cut ends. This assumption was used to explain the similarity of results with submerged and with suspended bundles: in both, the sugar entered at the cut end and moved longitudinally.

To test this hypothesis further, the authors [Kursanov & Turkina (63) selected material not involving the presumed effect of a bundle sheath. Narrow strips of bark of one-year old branches of *Caragana arborescens*, 5 cm. long, were found to respond with intensified respiration only when they were suspended with one end in the sugar solution. When they were completely submerged no increase in respiration resulted: sugar could enter through the entire exposed surface—which was assumed, without microscopic examination, to be composed of phloem—and no energy was expended in moving the sugar longitudinally.

Even if the bundle sheath could be regarded as a barrier against the entry of sugar, one might question whether only the presence and absence of a sheath explains the difference in results with *Plantago* and *Caragana*. The vascular bundles of *Plantago* contain xylem. *Caragana* bark strips contain no xylem, but have a considerable amount of parenchyma with chloroplasts, possibly even exceeding the active phloem in volume. Thus the systems used differ considerably. Furthermore, the authors did not actually determine whether the sugar moved upward in the suspended pieces of bundles or bark.

In any experiment involving the cutting of phloem, e.g., the use of stem segments, the possibility that plugging of the sieve tubes at or near the wound region may occur should be considered. The plugging may be the result of callose accumulation on the sieve plate with constriction of the connecting strands (Schumacher, 82), or it may be a coagulation of sieve-tube sap and slime-plug formation (Crafts, 19). Perhaps all three factors are involved. In any event, the introduction of solutes into the phloem across wound borders would seem to be more difficult than is sometimes assumed.

Results of studies on movement of nitrogenous substances were also interpreted to mean that translocation was dependent on cytoplasmic activity. The movement of asparagine and certain amino acids was followed in upper stem parts of excised wheat and rye shoots bearing immature ears but no leaves [Kursanov & Zaprometov (66)]. The excised shoots, 15

XUM

and 33 cm. long, were placed with the cut ends in the solution, or in water for control. Increase of total nitrogen, as compared with the controls, in different parts of stem and ear at certain time intervals served as the measure of movement of nitrogen. Most of the nitrogen rapidly accumulated in the grains.

The phloem was assumed to be the path of the translocated nitrogen because a comparison of absorption of water and accumulation of nitrogen suggested a more intense entry of nitrogen than could be explained on the basis of passive absorption with the water and movement upward in the transpiration stream.

Assuming that the translocation occurred in the sieve elements, the authors calculated the rate of movement in these cells as follows. Phloem occupies not more than 10 per cent of the transectional area of the wheat stem, the sieve elements only 15 per cent of the phloem area. (This value for sieve elements was taken from Schumacher's calculations pertaining to *Pelargonium*, a value actually not applicable to wheat.) Thus, a transection of wheat stem has 0.05 mm.^{2} of sieve elements. In 30 min. 2 mg. of nitrogen passed through this area. This amount corresponds to 12.5 mg. of asparagine. The concentration of the asparagine solution was 0.5 per cent. The authors think that, if mass flow were involved, the solution moving in the sieve tubes would be of the same concentration, a concept not implied in the mass-flow hypothesis. With these assumptions as a basis the authors obtained a value of 170 cm. per min., a rate considered to be in excess of all "real possibilities" in view of the resistance offered to the movement of a fluid by the narrow sieve elements filled with living substance.

The relation of absorption and movement of nitrogen to cytoplasmic activity was the object of a subsequent study [Kursanov & Zaprometov (67)]. The cut ends of excised upper parts of wheat stems, 30 to 35 cm. long, and deprived of leaves and immature ears, were placed to a depth of 1 cm. in a 0.5 per cent solution of glycine or asparagine. The submerged end was either the morphologically lower end, or the upper. After 20 min., pieces 5 cm. in length were cut off both ends of each stem piece and tested for increase in amount of nitrogen as compared with controls held in water. The result was that the accumulative power for nitrogen was stronger at the morphologically upper end regardless of which end of the stem piece was inserted into the solution. This observation was interpreted as evidence of a polar movement in the wheat stem at the particular stage of development considered. As to the tissue wherein this movement occurred, the authors suggested that any living tissue is probably capable of polar transport but that the sieve tubes may be specialized in this respect.

The studies on the various aspects of translocation led Kursanov (60) to conclude that the conducting tissues of plants are able to carry on not only active oxidation-reduction reactions, but also many of the reactions involved in carbohydrate-phosphorus metabolism. He postulated that these activities mobilize the energy required for the transfer of molecules. In the numerous

XUM

studies upon which these conclusions are based, however, no experiment was designed to localize the movement or the reactions to the phloem tissue specifically, and the deductions regarding this tissue rest on assumptions that in complex systems, such as vascular bundles or the bark of stems, the phenomena observed are determined by one of the components of the system, namely the active phloem. Even if one were justified in drawing conclusions about the phloem from the experiments described, the phloem is a complex tissue in which the reactivity of the various parenchyma cells may be expected to be different from that of the highly specialized enucleate sieve elements.

Conclusions

The student of phloem translocation is confronted today with several hypotheses concerning possible transport mechanisms. These can be described briefly: (*a*) mass or pressure flow [Münch's original hypothesis, (72)] embodying a physical movement of solution along turgor gradients through the continuous phloem system connecting leaf and root; (*b*) mass flow together with activities of parenchyma cells associated with the phloem that account for the turgor gradients necessary for mass flow [cf. Bauer (4); Crafts (24)]; (*c*) transport of solutes in the sieve tube along protoplasmic interfaces [van den Honert (95); reemphasized by van Overbeek (98)]; (*d*) accelerated solute movement in sieve tubes resulting apparently from some special kind of cytoplasmic movement or flow [Curtis (27)]; (*e*) independent solute movement resulting from one or more as yet unknown active transfer processes that occur in the sieve-element cytoplasm [Kursanov (60); Mason & Phillis (70); Schumacher (84, 87)].

There are many indications that the overall process of phloem translocation is metabolically controlled. Reference is made here to control that is more directly associated with transport than are such basic activities as production of assimilates and maintenance of differential permeability. Mass flow as usually understood today is a process limited to the flow of solution in the sieve tubes ("conduits") which, in a general sense, are surrounded by parenchyma cells ["pumps," according to Bauer (4)] having the capacity for active transport of solutes into or out of the conduits. This modified Münch hypothesis is supported by many types of observations, but is unconvincing as an explanation for certain aspects of translocation. For example, it is difficult to understand how bulk solution can move so rapidly across the sieve plate through the relatively small connecting strands of protoplasm.

Transport of solutes in the sieve elements along protoplasmic interfaces is a provocative explanation for movement of substances in the phloem, but it has not been demonstrated that protoplasm in the sieve elements meets the structural requirements of this hypothesis.

With regard to special types of cytoplasmic movement, the only visible evidence that it can occur is that reported by Rouschal (78) and by Currier

XUM

et al. (25). The evidence consisted of a "surge" of cell contents resulting from an artifically applied diffusion pressure gradient. It may be possible, of course, that special movement of a submicroscopic nature occurs.

Those workers who adhere to the explanation based on an active transfer process residing in the cytoplasm of the sieve element itself must envision enzymatic reactions that will support such active transfer of independently moving solutes. Arisz (2) considered it probable that every substance moves its own way, and that different mechanisms may be involved depending upon the type of solute transported.

While the dependence of translocation on a supply of metabolic energy cannot be doubted, it is not known just where or how the energy is utilized. With regard to the pressure-flow mechanism, the energy-requiring process (secretion of sugar across membranes) necessary in developing turgor gradients most likely resides in parenchyma cells associated with sieve tubes. As was mentioned before, Bauer (4) locates such "pumps" mainly in the leaf blade, specifically in the parenchyma cells of the fine veins.

The occurrence of strong acid-phosphatase activity in phloem and border parenchyma of veins [Frey (38); Wanner (101); Bauer (4)], although suggestive of secretory activity, is not a unique situation. This enzyme is widely distributed in plant tissues [Frey (38)] and is a part of the metabolic machinery of all cells. More detailed information is accordingly needed before it can be conclusively proved that active transport into the sieve tube is a function of such an enzymatic system.

In the animal, alkaline phosphatase seems to be associated with secretory cells [Moog (71); Rosenberg & Wilbrandt (76)]. Stepwise phosphorylation-dephosphorylation may account for transport of sugars across membranes [Danielli (28)] or even a "membrane of cells" in the kidney [Taggart (92)] and across the yeast cell membrane [Rothstein (77)]. In addition, an entirely different use of energy released through phosphatase activity should be mentioned. As Goldacre (42) and Danielli (28) have proposed, such energy may be utilized in the folding and unfolding of contractile proteins, a process which conceivably could result in accelerated transport of diverse kinds of molecules. Such utilization of phosphate-bond energy in the sieve-element protoplasm itself is difficult to visualize if Wanner's (103) report on the paucity of enzymes in sieve-element sap is correct. It may well be that such sap (phloem exudate) is not representative of normal sieve-element contents.

It is premature to select one and to discard completely all other hypotheses of phloem function. Any hypothesis must take into account and critically evaluate a large number of observations—the rapid exudation from the sieve tubes of cut stems; the surging flow of sieve-tube contents across sieve plates; the unique anatomical characteristics of the mature functioning sieve element, including the universal presence of callose in sieve areas; the direct histochemical evidence of the low metabolic activity in sieve elements; and the apparent common movement of assimilate (sugars) and widely divergent types of substances, e.g., dyes, auxins, viruses. It must account

XUM

for the inhibiting effects of certain poisons, low temperature, and anaerobiosis. It must consider the apparent differing rates of simultaneously applied tracer substances and the reported simultaneous movement in two directions.

The recent trend toward the application of biochemical, histochemical, and electronmicroscopic methods in studying phloem transport should be encouraged. Information is needed on the functions of the parenchyma cells; the nature, synthesis, and functions of callose and of slime; and the intimate structure and activities of sieve-element cytoplasm, especially those of the connecting strands and other kinds of plasmodesmata. Improvements in techniques to be sought include use of more suitable tracer substances, reduction of injury resulting from cutting and excising, more satisfactory methods of introducing solutes into the phloem, and methods for studying movement in individual uninjured sieve elements.

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XUM

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XUM

PHYSIOLOGICAL ECOLOGY[1]

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The fields of physiology and ecology are so closely interrelated that it is often difficult to detect any boundary between them. Along this ecotone lies the subject matter of physiological ecology (or ecological physiology)—as broad and diverse a subject matter in biology as it is possible to imagine. The diversity is so great that it is patently impossible to present a complete review in so short a space. Rather, it is the intention of the writer to try to convey some of the things which are of interest right now to ecologists and which have much promise in the future of physiological ecology and ecology in general.

Growth and Development of Individual Plants in Natural Environments

Germination studies.—The initial biological hurdle of germination in natural environments has been receiving considerable attention from ecologists and physiologists in recent years. [See Toole *et al.* (116)]. Because of the often long period of dormancy, the seemingly erratic germination of seeds of desert plants has been studied in some detail. Went and his co-workers (59, 139, 140, 141) found that germination in the southern California deserts depends largely on the temperature following sufficient rainfall. There will be no germination if the temperatures are too low or too high; winter annuals germinate at temperatures ranging from 8° to 18°C. and summer annuals and shrubs at temperatures of 26° and 27°C. There is a clear separation of the winter and summer floras as a result of these germination temperatures. *Pectis papposa* germinates only after being soaked in running water or after heavy rains (over 25 mm.). Went concluded that this is due to the necessity of leaching water-soluble germination inhibitors from the seed; but no evidence was presented as to what these inhibitors might be. Conversely, Koller (64) found that thorough washing of seed of *Calligonum comosum*, another desert species, completely inhibited germination.

Went found considerable irregularity in the germination of seeds of certain species, some viable seed failing to germinate with the majority but doing so after being subjected to other conditions. This apparently inherent characteristic variation could, of course, have considerable survival value in a rigorous and unpredictable environment.

Bünning (23, 24, 25), Bünning & Müssle (26), and Koller (65) reported yearly, apparently endogenous, rhythms in the viability of certain seeds. However, Went and his colleagues were not able to detect such rhythms in their experiments. The separation into warm- and cold-germinators ap-

[1] The survey of literature pertaining to this review was concluded in October, 1956.

XUM

peared at any time of the year providing the proper temperature conditions were satisfied.

That the germination-regulating mechanisms in desert seeds are complex and that they vary even within the same genus is apparent from the work of Beadle (1) and Koller (65) with *Atriplex*. Both found that germination was inhibited by the presence of the surrounding bracts. Beadle ascribed this inhibition in five Australian species to the presence of the chloride ion in the bracts, while Koller with *A. dimorphostegia* from the Negev Desert indicated that chloride was not directly responsible. However, while Beadle and Koller are in apparent disagreement, both appear to mean that it is primarily the osmotic effects of chloride which are the principal causes of inhibition. Koller believes that there may be other, possibly organic, substances involved in the inhibition also. *A. dimorphostegia*, unlike the Australian species, is light-inhibited in its germination.

The discovery of the reversible photoreaction in the germination of lettuce seed by Borthwick *et al.* (17) is of great significance to ecological germination problems. Toole *et al.* (117, 118) found the reversible photoreaction in seeds of many of the higher plants, but it is not always necessary for germination. The substances and processes which the photoreaction controls are also under the control of temperature. This new information indicates the complexity of seed germination in particular regard to patterns and combinations of light of various wavelengths, temperature, and water; ecologists, particularly, should be extremely careful of light quality—temperature regimes in germination studies of native species of any of the higher plants. The possibility of germination inhibition by stray infrared casts doubt on germination percentages in many recent studies and makes it imperative that the investigator control the quality of light as well as the intensity and photoperiod. Such environmental germination studies of the component species of desert and semidesert vegetation could be of considerable value in the interpretation of the characteristically wide yearly variations in the composition of such vegetation.

Microclimatic studies and phenology.—With the development of new instruments and techniques in microclimatic analysis in recent years [Geiger (44, 45); De Backer (38)], there are at present a number of intensive investigations of plant microenvironments in process. Coupled with this is a renewed interest in the old subject of phenology. Starting with the work of Sørensen (109) on arctic plants, the last fifteen years have seen rapid developments in the correlation of plant growth in the field with meteorological events and cycles. The next five years should be quite productive along these lines.

The importance of realizing the great variation in microenvironments arranged both vertically and horizontally in even a small area has been emphasized by Wolfe *et al.* (146). It is often completely misleading to attempt correlations with weather data gathered even a short distance away from field experiments unless standard weather data are also gathered at the

XUM

actual site of work. This is even more applicable to the temperature situations near the soil—atmosphere interface as shown by Geiger and emphasized in the recent works of Cantlon (27), Billings *et al.* (7), Maguire (77), and Bliss (15).

Bliss, in particular, has made careful correlations between the climate near the ground in arctic and alpine tundras and phenological events of the highly specialized tundra plants. He measured stem elongation and leaf expansion of a number of species in several kinds of topographical situations while instruments continuously recorded the factors of the physical environments at the plant levels in the same locations. He concluded that higher air temperatures near the ground and soil temperature were most influential in governing the rate and amount of plant growth of tundra species in northern Alaska. However, the cessation of plant growth occurs when temperatures are still adequate and thus it appears to be caused by the interaction between some other factor of the environment and the genetic constitutions of the various species. Temperature also was apparently the most important factor in the growth of alpine plants but the degree of correlation was much lower than with arctic species. He concluded that, in general, the alpine environment is more severe than the arctic environment during the growing season because of the shorter photoperiod and its effects on nocturnal terrestrial radiation and temperature fluctuations. Stronger winds, greater aridity, and higher ultraviolet radiation add to this severity.

Lindsey & Newman (76), attempting a correlation between official weather data from a nearby station and a thirty-year spring phenology record in northern Indiana, also found a high degree of correlation between early growth and temperature. They devised a new temperature summation method, based not on means but on daily maximum and minimum temperatures, so that the approximate durations of different temperature levels during the diurnal cycle are emphasized. These duration-summation values and time periods for the flowering of 24 species were analyzed by punch card machines for standard deviation and coefficient of variation so that the requisite heat sum for flowering was found for each species. These ranged from 2,034 degree-hours for an early species to 8,533 degree-hours for a late-flowering species.

A great need in field plant growth studies is the development of accurate portable instruments for the measurement of physical environmental factors which can be used under severe conditions and without being dependent upon an external power source. Some, such as the integrating light recorders developed by Somers & Hamner (108) and Blackman *et al.* (14), are in use now but others are needed. An inexpensive thermistor recorder would be especially useful.

Photoperiodism and plant distribution.—The effect of photoperiod on the distribution of species and genera is fairly well known. The existence of photoperiodic strains or ecotypes has been demonstrated for the grass *Bouteloua curtipendula* by Olmsted (95). The relationships between photo-

XUM

period and the physiology and resultant distribution of boreal species of trees and herbs has been receiving considerable attention and is a fertile field of investigation for the ecologist and forester. [See Wareing (131)].

Wareing (127, 128, 129), working with *Pinus silvestris*, found that the greatest height growth and leaf number in first year seedlings occurred with a daylength of 20 hr. After the first year, the number of nodes is pre-determined by the number of initials laid down in buds of the previous year, so the duration of growth cannot be affected by photoperiod. Nevertheless, extension of internodes is affected, being reduced under short daylength. The effect appears to be direct and not through the leaves as is the flowering stimulus in herbs.

Wareing (130) showed that no chilling period is necessary to break dormancy in the buds of European beech (*Fagus sylvatica*). Seedlings kept leafless through the winter in the dark at 15°C. broke dormancy when exposed to long photoperiods at 20°C. but remained dormant in photoperiods of 12 hr. or less. The intensity of light needed to break dormancy if the photoperiod is right is rather low; only 1,000 lux is needed to fully saturate the light requirement of intact buds. Only 0.7 per cent of the incident light penetrates the bud scales. No more than 7 lux, then, are actually needed in the meristematic region to start bud-break. The response of the buds seems to be governed more by the length of the dark period rather than by the length of the photoperiod. Wareing suggests that the action of the growth-promoting light period and the inhibitory dark period might be similar to that in light-sensitive lettuce seeds [Borthwick *et al.* (17)].

Black & Wareing (8) did find Borthwick's reversible photoreaction in *Betula pubescens* seeds, with infrared inhibiting germination. If infrared exposure was allowed within 10 hr. after red light exposure, the germination process could be stopped. After 12 hr., however, germination could not be halted by infrared. While chilled *B. pubescens* seed have no light requirement for germination, unchilled seed need light at 15°C. Long days result in higher percentages of germination than short days. At 20°C., the photoperiodic nature of the response is lost and high percentage germination occurs under both long and short days. Vaartaja (122) reported essentially the same response for unstratified seeds of this birch. However, at temperatures over 25°C., no light was necessary for maximum or nearly maximum germination.

There is every reason to believe that wide-ranging tree species are made up of many ecotypes which differ in their photoperiodic reactions as did Olmsted's races of *B. curtipendula*. Sylven (113) as early as 1942 discovered such ecotypes in the European aspen. Karschon (60) found, within the same photoclimate of the Alps, strains which reacted differently to photoperiod depending on the elevation of the seed source. Vaartaja (121) demonstrated northern and southern photoperiodic ecotypes in *P. silvestris* and *Alnus incana* in Finland. In 10 hr. photoperiods, plants of both northern and southern races grew rather poorly, (the northern ones more so than the southern) but under continuous light there was a great increase in the growth of the

northern plants as compared to that of the southern plants. Wassink & Wiersma (132), also working with *P. silvestris*, found that seedlings from seed sources in France (45°30′ N) grew very well in long days (24 hr.) as well as in short days (12 hr.). On the other hand, seedlings from a Swedish seed source (66° N) did very well in the long photoperiod but very badly under short day conditions. In view of these results, it is somewhat surprising to note that Downs & Borthwick (39) compared photoperiodic reactions in several species of trees, each from only a single seed source, with the seed sources ranging from Maryland and Michigan to Sweden, and with the source completely unknown for *Pinus taeda*. They do, of course, realize that photoperiodic ecotypes exist and concede that discrepancies may be due to the use of different ecotypes in different studies.

Resende (100) discovered that several species of plants require both long-day and short-day induction for blooming. He termed these "long-short day plants." Recently, Sachs (104) has shown that *Cestrum nocturnum* (night-blooming jasmine, a Solanaceous shrub from the West Indies) is a long-short day plant. With *Cestrum* as a model, Sachs suggests that floral initiation by intermediate day plants may be controlled by two overlapping photoperiodic reactions. More information of this sort may help to explain the distributions of certain subtropical plants.

Many arctic-alpine species grow over a very wide latitudinal and, thus, photoperiodic range. It is regretable that so little physiological work has been done with these extremely specialized plants. Wilson (145) has worked with *Oxyria digyna*, a widespread arctic-alpine species, at Jan Mayen (71° N) in its native environment and also on transplants in England from the Jan Mayen source. Even under continuous daylight in midsummer on Jan Mayen, there was a clear diurnal stomatal movement with the stomata being more widely open during the day than during the "night." The amplitude of the movement was only about one-fifth of that found in the *Oxyria* grown in England, however. Total leaf carbohydrate (sugar plus starch) was about twice as high in the Arctic as in England. Total sugar showed no diurnal rhythm at Jan Mayen but a clear one in England, while the diurnal variation in starch content was more than twice as great under arctic light conditions than under temperate ones. Constant illumination, therefore, under arctic environments does not necessarily result in lack of diurnal rhythms. Much more information is needed on the physiological processes of arctic and alpine plants before their adaptations to severe environments can be understood. It would be profitable to compare the physiological ecology of arctic ecotypes with that of alpine ecotypes of the same species. Growth chambers (small "phytotrons") similar to that devised by Fisher (42) for his work on alpine *Ranunculus* species in New Zealand would be most helpful in this type of investigation.

Water relations.—Probably no other ecological factor has been studied as much as water with its effect on plant growth and distribution. Among the several ecological reviews on the subject, the recent paper by Troll (119) is

XUM

particularly interesting. Many shorter papers are of ecological importance but a few will serve to illustrate some intriguing ideas.

For a number of years, the value of dew as a source of water in the environment has been debated. Breazeale and his co-workers (20, 21) reported the absorption of water from a fogged atmosphere by leaves of plants and its exudation by roots into flasks and soil. They evidently did not keep the root environment at a constant temperature, however. This could result in accumulation of water in the flask due to evaporation from root surfaces and subsequent condensation on the sides of the flask due to cooling. Haines (50) attempted to duplicate Breazeale's results under constant temperature and could not find any evidence of exudation per se. He did agree that tomato plant leaves could absorb water from a saturated atmosphere, but thought that the water in the flask resulted from condensation because of temperature changes and resultant flow of water down through the plant along a DPD (diffusion pressure deficit) gradient. He got similar results using a cotton wick instead of a tomato plant.

Janes (58), growing his plants in soil, also could find no evidence of exudation from roots even though the leaves were in a saturated atmosphere; eventually the soil moisture dropped to the wilting percentage. Stone & Fowells (111) found that simulated dew from fogging allowed seedlings of *Pinus ponderosa* to survive longer in soil at or below the permanent wilting percentage, but there was continued loss of water from the soil through the plants and no evidence of exudation. They ascribed better survival in fog to the lower moisture stress under which the plants were growing. In further work, Stone and his colleagues (112) found that it was definitely temperature fluctuation in the flask that allowed water to move from a saturated atmosphere through the leaves of pine and out of the roots into the flask atmosphere. In other words, this movement is apparently attributable to a vapor pressure gradient just as Haines concluded.

This gradient appears to be so low under soil conditions at constant temperature that the slow movement of water from the air to the soil through the root "wick" would be extremely slow. However, there is another aspect to this problem of a plant's ability to survive under desert soil conditions and that is the fact that the surface soil in a natural environment is not at a constant temperature by any means. Billings *et al.* (7) found that in the deserts of western Nevada there was almost always an amplitude of 50 to 70°F. in the diurnal temperature cycle at one inch under the soil surface for almost the entire dry season. This could possibly result in some moisture condensation at night in the surface soil, assuming a vapor pressure gradient from moister layers deep in the sandy soils. Boyko & Abraham (19) believe that shallowly rooted *Amaranthus blitoides* in the eastern Mediterranean region gets some of its water from such subterranean condensation (but perhaps not from below), although they credit dew on the leaves as another source during the dry summer season. Apparently dew can aid survival, but it would be most helpful to have more data on soil vapor pressure gradients

and the possible absorption of water by the roots of desert plants from saturated or near-saturated soil atmospheres.

Another facet of the fog-dew condensation problem is that of fog drip from trees with resultant increase of soil moisture. This has been reported from California by Means (84), Kittredge (62), and Oberlander (89) to increase greatly the effective precipitation and resultant soil moisture under certain kinds of trees. The exposure of the tree in regard to the direction of fog movement makes great differences in the amount of drip. Essentially the same thing occurs in Hawaii as the result of condensation from trade-wind clouds on the leaves of planted Norfolk Island pines (*Araucaria excelsa*). This is particularly prominent on the Island of Lanai where there are current investigations of its hydrological significance. Such a phenomenon may be of considerable ecological importance in the forests of mountainous regions with much cloud or fog.

The use of soil moisture by vegetation and crops in relation to potential and actual evapotranspiration has been studied recently by Zahner (147) and Slatyer (107). Zahner found that actual and potential evapotranspiration were approximately equal in southern Arkansas. There was no significant difference between that occurring from a pine forest and that from an oak forest. This supports theories that in a moist region evapotranspiration is independent of vegetation type. On the other hand, Slatyer has clearly shown that in northern Australia actual evapotranspiration from a field of grain sorghum is quite different from that of peanuts or cotton during a long dry season following heavy rain. Because of its deeper and more extensive root system, actual evapotranspiration from sorghum remains close to the potential for a longer period of time before decreasing rapidly with decline in available soil moisture.

A real necessity in these ecological problems of water use is the development of good field methods for the measurement of actual transpiration as affected by environmental factors. The Thermoflux apparatus described by Huber & Miller (56) appears to be very promising in this regard. The amount of transpired water vapor is determined from temperature measurements of sulfuric acid before and after an airstream from the transpiring plants is passed through it.

Parent material effects.—In the last few years, it has become recognized that the type of underlying rock or parent material is usually fully as important in determining plant distribution and vegetational pattern as is climate. The importance of the edaphic factor and its strong selective action in the evolution of endemic species and ecotypes has been emphasized by Mason (78, 79) and by Mason & Stout (80).

The vegetation of serpentine is usually so strikingly different from that of other rocks in the same climate that it has been the subject of considerable study. The papers of Kruckeberg (68, 69), Walker (124), Whittaker (142), Walker *et al.* (125), McMillan (83), and Tadros (114) discuss this problem thoroughly, particularly as it appears in American West Coast vegetation.

XUM

In addition, Rune (102) emphasizes the importance of this rock type in the distribution of arctic and alpine plants.

Serpentine is essentially a magnesium silicate. Walker (124), using both ordinary cultivated plants and native serpentine endemics, found that it is apparently the low calcium level of serpentine soils which is the principal limiting factor. Addition of calcium to serpentine soil had a far greater effect on the growth and yield of tomato than it did on the yield of *Streptanthus glandulosus* var. *pulchellus*, a serpentine endemic. Native species which do not invade serpentine reacted much as did tomato. Walker believes that a tolerance to a low calcium environment offers the best physiological explanation of serpentine tolerance. In addition, the serpentine species must be tolerant of one or more of the following conditions: high concentration of nickel and chromium, high magnesium, low levels of major nutrients, and low available molybdenum.

Kruckeberg (68, 69) found that "bodenvag" species of a single climatic ecotype which can grow either on or off serpentine are able to do so because their serpentine populations are adapted to low calcium levels. In most cases, therefore, the serpentine population is ecotypically distinct from that on nonserpentine soils, even though morphologically similar, and constitutes an edaphic ecotype within a climatic ecotype. Kruckeberg believes that serpentine plants may be restricted to serpentine by the more rigorous competition in the nearby normal communities. This introduces a biotic factor into the serpentine problem. Tadros (114), working with *Emmenanthe*, has suggested the possibility that it may be soil microorganisms in nonserpentine soil which prevent the establishment of certain serpentine tolerant plants on more fertile soils. These microorganisms would supposedly be kept out of serpentine soils by the low calcium levels.

Whittaker (142) has described the stunted xerophyllous serpentine vegetation of pines and other conifers, sclerophyllous shrubs, and grasses in the Siskiyou Mountains of Oregon as contrasted with the nearby dense *Pseudotsuga* forests on diorite. He ascribes the scrubby serpentine vegetation to poor productivity in this type of ecosystem attributable to low nutrient levels (especially calcium) in the soil. He quite rightly concludes that the vegetation of these serpentine areas is climax in the same sense that the forest on diorite is climax.

Extremely acid hydrothermally altered rocks in desert climates of the Great Basin usually are occupied by pines and other montane (or endemic) species. Billings (4) and Salisbury (105) have found that these soils are quite deficient in phosphorus and nitrogen and that certain native species can tolerate low levels of these elements while most species cannot. Low phosphate appears to be the principal limiting factor. Salisbury showed, using mass law considerations, that for the iron-phosphate system in such soils, simple equilibrium conditions prevail. Therefore, low phosphate is the result of high iron, aluminum, and other ions at acid pH.

The effects of low phosphate in delimiting vegetation types have also been demonstrated by Beadle (2) in Australia and McMillan (83) on acid

soils in the Coast Ranges of central California. In Australia, phosphate-deficient soils are occupied by sclerophyllous vegetation while the adjacent normal soils support rain forest.

The vegetation of these phosphate-deficient areas is not successional but climax; the chemical nature of the parent rock cannot be overcome by the climate. It is important to know why certain species of *Pinus* and *Cupressus* in North America and certain kinds of *Eucalyptus* in Australia can grow on these very poor soils at all. There are all too few mineral nutrition studies on wild plants of any kind. Endemics of this type, either at the species or ecotype level, must have a really efficient mineral-gathering method or some other metabolic compensation. In the evolution of regional floras and vegetations, such physiological adaptations would have high survival values under the selective effects of extreme types of parent materials.

Competition.—The concept of "competition" is one being debated at the moment. Is it a real thing or not? If it is real, what is it? How does it operate? There is need for a critical examination of the concept.

Mason & Stout (80) criticize the concept of "competition," as generally used, as being so all-embracing that the term becomes meaningless. They suggest restricting it to cases in which actual direct inhibition has been demonstrated. Such a demonstration of inhibition under field conditions is difficult to make, and actually direct inhibition may be a rare thing even though it does occur. Gray & Bonner (48, 49) and Bennett & Bonner (3) have found that the leaves of certain American desert shrubs (*Thamnosma montana*, *Encelia farinosa*, *Franseria dumosa*, and others) contain substances toxic to tomato plants in culture. As Muller (87) and Muller & Muller (88) point out, however, certain herbaceous plants such as *Malacothrix*, *Chaenactis*, and *Cryptantha*, even though inhibited in laboratory experiments by such leaf substances, do grow under these very shrubs under desert conditions. Therefore, they conclude that there appears to be no competitive advantage as a result of the production of such toxins.

On the other hand, Keever (61) found that roots of *Leptilon canadense* (horseweed) prevented growth of seedlings of horseweed and thus were a factor in the short-term occupancy of abandoned land by this species in Piedmont succession. Osvald (97) and Hamilton & Buchholtz (51) have shown that living rhizomes of *Agropyron repens* inhibit the germination and growth of the seedlings of most weedy species but promote the emergence of some others. Ooyama (96) has reported that a water extract of leaflitter of certain Japanese conifers inhibits seed germination and seedling growth of the same conifers and other species.

These, then, are concrete examples of actual biostatic inhibition and certainly others could be cited. The phenomenon appears to be rare among the higher plants but perhaps the rarity is illusory simply because of lack of data—it may be widespread. Bormann (16), using tracers, has shown that phosphorus and water added through the soil to one plant may be carried through its roots to new soil areas and appear in the tissues of adjacent plants. It is conceivable that organic toxins, growth substances, or toxic

XUM

ions could be carried and released by the roots of certain plants in the same manner.

Direct inhibition may be somewhat of a rarity but indirect inhibition through the environment certainly is not. It is common knowledge that growth in a very dense stand of pine, for example, is inhibited for all of the individuals concerned because of depletion of certain environmental factors at certain critical times.

Both environmental and genetic factors are involved in the effect of competition on survival in times of stress. In a pure stand of a single biotype, it is environmental variation which determines which individuals will grow better and survive better. In a mixed stand in an areally (but not chronologically) uniform environment, it is the more perfectly adapted biotype which survives critical periods. If a pure stand occurring in an areally uniform environment is subjected to severe environmental stress, all of the individuals may be stunted or killed because their genetic tolerance demands the same environmental fulfillment at the same time. This can happen with pure stands of forest trees, grasses, or weeds, and often results in sudden replacement by new dominants more in adjustment with the environmental cycles. However, without environmental stress the reproductive capacity of some species is self-balancing over a wide range of plant densities [Hodgson & Blackman (54)]. In their work with *Vicia faba*, they found that small crowded plants produced just as many seeds as large widely spaced plants. Certainly there are limits beyond which this would not be true.

The more genetically diversified a population of a species is, the more chance that species has of being represented in the resultant community after years of "competition" or environmental selection pressure. If a species or ecotype has been successful in "competition," i.e., has survived through critical times in mixed vegetation, it must have some key characteristic with survival value. Yet usually the physiological trait and how it works is not known. Kramer & Decker (67) and Kozlowski (66), however, have shown some of the reasons why pine is relatively unsuccessful in Piedmont forest succession while oaks become the eventual climax dominants. Photosynthesis is reduced more in pine by shading than it is in oak. This results in a reduced root-shoot ratio, reduced carbohydrate content, and reduced growth in pine. The result is that, under a pine overstory, pine seedlings with shallow and weakened root systems cannot survive summer drought in the upper part of the soil. On the other hand, oak seedlings with greater food reserves, greater photosynthetic ability, and deeper root systems can survive and eventually, almost inevitably, replace the old pines.

The climax oak forests themselves are of several types depending on parent material variations. The composition of each of these types is dependent not only upon the mineral requirements of the various oak and hickory species but also on the ability to "compete" or survive minimal factors in a biotic community. Bourdeau (18) has demonstrated that seedlings of certain oak species from the better sites where the forest is dense can carry on maximum photosynthesis at much lower light intensities than can those from

the more open poorer sites. This allows ample reproduction of such species to the exclusion of the more light-demanding species from poor sites. Conversely, oaks from the good sites cannot invade the poor ones because of insufficient drought resistance as compared to the usual dominant species in such locations.

That the composition of the plant community affects the course of competition or environmental selection, is evidenced by the results of Ellenberg (41), Lazenby (72, 73), Wilson & Peake (144), and Holmgren (55). Ellenberg (41) has shown clearly that many species can grow very well in conditions thought to be beyond their tolerance ranges if competition is removed and that the physiological optimum obtaining in pure culture is by no means the same as the ecological optimum in mixed stands under field conditions. These points have been further emphasized and amplified by Knapp (63) and Walter (126).

Integrated ecological life histories.—The importance of genetically determined tolerance ranges of species in governing potential geographic range is generally accepted, in theory, at least [Good (46)]. Very little, however, is known about the actual tolerance ranges of any wild species with a few notable exceptions. Tolerance range work must not only concern itself with the laboratory determination of factors limiting growth but also with the plant populations of the species as it grows in nature.

An outstanding example of this much needed type of study is that done with *Scilla non-scripta* in England by Blackman and his co-workers (9 to 13). By intensive laboratory and field experiments, they know almost exactly where the various ecological strains of *Scilla* will grow in England, and even more important, why they will and do grow there.

Watt (133 to 138) has made similar studies of *Pteridium aquilinum*, the bracken. Since *Scilla* can grow with bracken, there would seem to be an excellent opportunity to study the reciprocal ecological relations of two very well-known species.

The work of Hulbert (57) on the ecology of *Bromus tectorum* (cheatgrass) and other members of the genus in western North America is a start toward the understanding of the ecology of a genus. This kind of comparative ecology of related groups of species is useful not only from the standpoint of pure plant geography but also has considerable practical value to the forester or range manager.

Comparative ecology of related taxa.—Since the ecological ranges of species and genera are determined by the tolerance ranges of their component ecotypes or species, respectively, one should know how much these ecological requirements vary from one component of a taxon to another. This comparative ecology must have its foundations in genecology [Constance (35)] and use all the techniques of physiology, ecology, etc. But, perhaps, the real need is to recognize that a species or a genus is a complex of many ecological races each made up of somewhat variable local populations. Ecological or physiological work on a single strain or local population of a species cannot be extrapolated and considered as typical for the whole species, unless it is a

XUM

very narrow endemic. As stated by Clausen (30, 31), each local population of a successful species may consist of many genotypes each solving environmental survival in its own way. Excellent evidence for this tolerance variability within single populations of *Achillea* is given by Hiesey (52). The survival advantages of such variable populations in the normal fluctuations of climate are obvious.

Strangely enough, it is only in recent years that the classic works of Turesson (120) and Clausen, Keck & Hiesey (32, 33, 34) have had a real impact on ecology. Now that they have shown that wide-ranging species are made up of many climatic ecotypes, and that Kruckeberg (68) has shown that each of these may consist of several edaphic ecotypes (which in turn are made up of biotype-rich local populations), the complexity of such species in relation to their environments is beginning to be apparent.

Ecotypic variation can be shown with regard to almost any factor of the environment. Such variation is the result of environmental selection of mixed genotypic material of the local populations. Photoperiodic races have been demonstrated by Olmsted (95), Larsen (71), and McMillan (81, 82) in various grasses of the American prairie. There are also many reports of photoperiodic races in tree species [Sylven (113); Karschon (60); Vaartaja (121); Pauley & Perry (98); and Wassink & Wiersma (132), among others]. There are also temperature ecotypes of many kinds [Hiesey (52) in *Achillea*, (53) in *Poa*; Cottam (36) in *Populus tremuloides*]. Moreover, it is well known that different horticultural varieties of a single species may react quite differently to different temperature climates, as does alfalfa [Bula *et al.* (22)]. Many examples of edaphic ecotypes have been mentioned in a previous section. Similar illustrations could be listed for other factors and other species but this is sufficient to demonstrate that natural communities are made up of physiologically variable species in which ecotypic combinations of all conceivable kinds may be found.

What is the significance of this in community ecology? The floristic composition and dynamics of the grassland communities of the American prairie are probably as well known as for any vegetation. McMillan (81, 82) took clones of five important dominant grasses of the prairie from five widely separated geographic locations where they were growing together. These ranged from northwestern Nebraska to southeastern Nebraska, a distance of about 450 miles. The grasses, *Bouteloua gracilis*, *B. curtipendula*, *Panicum virgatum*, *Andropogon scoparius*, and the *A. gerardi-A. hallii* complex, were grown in a uniform garden in Lincoln, Nebraska, and certain ones grown under different photoperiods. The western forms were the earliest-flowering and the eastern forms invariably flowered latest in the uniform garden. This early flowering of the western forms appears to be correlated with early onset of the dry season in the west and also with early fall frosts. This relatively severe environment has selected out the faster-growing and earlier-flowering individuals from the genetic pool of each species while the longer growing season in the east has favored the more slowly growing forms. Sørensen (110) has shown a positive correlation in certain biotypes of *Capsella* between early-

flowering and adaptation to a short growing season and poor nutrition. Such a situation might well exist with McMillan's plants since the soils of western Nebraska are somewhat poorer than the rich soils of the more eastern prairies. McMillan concluded that the presence of the same series of taxa does not necessarily afford a safe criterion for judging the habitats to be the same.

Whittaker (143) also discussed the indicator problem in the light of present knowledge of the complexities of the species, the community, and the environment. He criticizes the use of single species as indicators for single factors, pointing out that the complexity of the species and the complexity of the environment do not allow such simple cause-and-effect indicator interpretations. For practical purposes, however, he suggests weighted averages of community composition, empirically related to a given factor, as an effective indicator technique.

Growth, Development, and Productivity of the Ecosystem

Ecology, unlike physiology, must concern itself with the functioning of the total biotic community—environment complex or ecosystem [Tansley (115); Lindemann (75); Billings (5); Odum (90); Clarke (29); Sjörs (106)]. Organisms in nature are integral parts of ecosystems. Therefore, their physiological ecology must be considered as it is involved in the energy-flow through the system. It is not enough to realize that a species is made up of varied biotypes, that a biotic community has structure, that the environment is complex. One must also try to find out what makes the whole system work. What might be called "ecosystem physiology" is fundamental to this understanding.

Ecosystems are the basic self-sufficient units in nature. The ability of certain components (the primary producers) to introduce energy from solar radiation into the system by photosynthesis is the foundation of the complex biotic community. This energy flows through the food chain of the community with losses by respiration at each trophic level [Lindemann (75); Clarke (28)]. It is necessary to know what the production and loss is at each trophic level if the total dynamic economy of the system is to be understood. At the primary producer level, this means measurements of photosynthesis and respiration in natural communities as affected by light, temperature, nutrient supply, and other environmental factors. Once the energy is in the system, its production of biomass and loss through respiration must be followed at each trophic level. This approach has been emphasized by both Odum (90) and Clarke (29), and it seems to promise the most significant developments in the future of ecology.

Total photosynthesis and respiration are somewhat easier to measure in aquatic environments than in terrestrial environments. Food chains are also better known and may be less disturbed in aquatic environments. It is not surprising, therefore, that the recent rapid development of ecosystem ecology has been largely attributable to accelerated research with aquatic ecosystems. The recent contributions of Ryther (103), Odum & Odum (93), Verduin (123), and Edmondson (40) provide basic information of the needed

XUM

type concerning total photosynthesis and respiration in such ecosystems.

Odum & Odum (93) have shown that the production (mostly photosynthesis) on a windward coral reef at Eniwetok is just about balanced by the total respiration on the reef. Verduin (123) found the two processes in similar equilibrium in the aquatic communities of western Lake Erie. Thus, there is no net output, as in successional or agricultural communities, and the communities are in a steady state or climax condition according to the theory of Odum & Pinkerton (94). The system is in equilibrium, with all energy coming into the community being completely used by leakage and maintenance. While the total production of the Eniwetok reef per year was very high (24 gm./m.^{2}/day or 74,000 lb./acre/yr.), the average standing crop was low; the actual ratio of production/standing crop was 12.5, indicating a complete turnover in the community about once a month. This high productivity however was only about 6 per cent efficient in terms of incident solar radiation. This would be more or less in agreement with the hypothesis of Odum & Pinkerton (94) that the optimum efficiency in a highly productive system is low; the survival is the result of the high productivity and not the efficiency.

Productivity of uncultivated terrestrial ecosystems is not well known. While excellent agriculture may produce around 50 gm./m.^{2}/day, Billings & Bliss (6) found net productivity in an alpine tundra near a semi-permanent snowbank to be of the order of 2 gm./m.^{2}/day for about 40 to 45 days or about one-twelfth the daily productivity of the Eniwetok reef. Moreover, there is only one turnover per year as compared to 12.5 turnovers per year on the reef. In the alpine tundra, a low-producing system in a rigorous environment, it might be expected that survival of the producers is due to relatively high efficiency as contrasted to the low efficiency of the reef. Unfortunately, there are no data as yet to prove or disprove this. Odum (92) found net productivity on an abandoned field near the Savannah River to be about of the same low order of magnitude (ca. 1.5 gm./m.^{2}/day) as that of the alpine tundra, but the productive period extended over a much longer growing season than in the tundra (183 days). Certainly, measurements of this kind obtained to date are relatively rough compared to laboratory measurements. However, they are a start in the right direction.

The realization of the complexity of environment-biotic community relationships and the rise of "ecosystem ecology" has been more or less concomitant with the development of atomic energy uses. While relatively little is yet known about how ecosystems work, what is known comes none too soon. With radiation sources becoming increasingly more numerous, a knowledge of its pathways and accumulation points in the many kinds of ecosystems is badly needed. Conversely, radioactive isotopes can be used as tracers through wild plants and in the ecosystem [Melin & Nilsson (85, 86); Pendleton & Grundmann (99); Graham (47); Rigler (101)].

Within the last two or three years have come the beginnings of a new aspect of ecology, "radiation ecology," using all the tools of modern ecology, including a knowledge of the biological effects of radiation. The beginnings

have been on a small scale and often neglected in the great emphasis on laboratory experiment in radiation biology. Proper equipment, manpower, and controls have usually not been available. Nevertheless, there are the welcome contributions of Krumholz (70), Foster & Rostenbach (43), and Davis & Foster (37) on radiation problems in stream environments, Odum & Odum (93) on radiation in the Eniwetok reef environment, and Lindberg & Larson (74) on the ecological aspects of radioactive fallout. Odum's as yet unpublished work on radiation ecology in the ecosystems of the Savannah River Plant of the Atomic Energy Commission will provide more badly needed information. Odum (91, 92) has made excellent summations of the problems of radiation ecology up to the present time. It is imperative that we understand much more about ecosystems and their complexities, particularly in regard to radioactive waste disposal, if man is to live in a world with increased radiation sources, because man is a part of the ecosystem too.

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XUM

PHYSIOLOGICAL GENETICS[1,2]

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Physiological genetics can be defined as the study of the mechanism of gene action, including gene duplication. It is, as the name implies, a hybrid subject. The primary concern of geneticists is usually the mode of inheritance of characters, not the processes at a molecular level responsible for their expression. On the other hand, physiologists in their studies of plant growth and development mechanisms, often neglect potentially fruitful genetic approaches. It is the purpose of this review to consider examples of work carried out on higher plants where physiology and genetics have been jointly applied to the study of fundamental problems. Ultimately the mode of action and interaction of the whole gamut of genetic factors is the legitimate concern of physiological genetics. For the purposes of this review the limit will be arbitrarily drawn at examples of genetic determination where the characters have been studied from the point of view of their chemistry or physiology, or where related physiological work indicates something about the nature of the process involved.

Gene Effects and Genetic Methodology

For fundamental principles of inheritance and what is known about the nature of the hereditary units themselves, see the textbooks by Srb & Owen (1) and especially Wagner & Mitchell (2) and the recent review by De Busk (3).

It has been postulated by Beadle (4) that the self-reproducing hereditary units, the genes, bring about their effects through determining the specificity or activity of enzymes, one gene being primarily concerned with one enzyme. As discussed by Horowitz & Leupold (5), this does not imply that more than one gene cannot be concerned in the production of an enzyme nor that the phenotypic expression of a gene is restricted to a single effect or function. Lockingen & De Busk (6) have advanced an attractive scheme to account for the transfer of gene specificity to enzymes. According to their model gene-controlled enzyme specificity is determined by a template synthesis of specific ribonucleic acids.

If we assume that genes somehow produce their effects through determining enzyme specificity, a genetic difference of the *Neurospora* "blocked reaction" type can be represented as:

$$A \xrightarrow{\text{gene-controlled enzyme}} B$$

where a precursor or substrate molecule A is converted to product B through

[1] The survey of literature pertaining to this review was completed in October, 1956.

[2] Florida Agricultural Experiment Stations Journal Series, No. 580.

the activity of a gene-determined enzyme. The presence of the normal versus the mutant allele determines whether the product B is synthesized because it determines whether the enzyme is active. Such a picture, while it serves a useful function in elementary visualization of gene differences, may be overly simple in two important respects. First, the evidence indicates that mutations more frequently involve "somewhat changed" rather than "completely blocked" reactions, and therefore changes in the formation of B can be considered as changes in the rate of conversion of A to B. Second, a gene that influences the conversion of A to B need not operate by a direct effect on the enzyme, although the cases of gene-enzyme relationships that have been studied to date indicate that direct effects are common (7).

It is apparent that an altered rate of one cellular reaction is likely to influence other reactions. Wagner & Mitchell (2) pointed out that the various primary and secondary changes attributable to a mutant gene can be considered as determining an altered metabolic pattern. The secondary or alternate effects of a gene-induced metabolic change can be regarded as pleiotropic effects, that is, multiple effects of a gene. Any gene might appear to be pleiotropic if the change in metabolic pattern is sufficient to cause more than one observable change. Indeed, nonpleiotropic genes may merely be examples of genes where the secondary effects are not obvious. Pleiotropic genes, in the sense of true multifunctional genes, which determine the specificity or activity of more than one protein, may exist; however, Horowitz & Leupold (5) have advanced an ingenious argument from their work with bacterial mutants which indicates that if multifunctional genes occur they are not numerous.

If the primary or secondary effects of one mutant gene differentially influence the expression of nonallelic genes, the first gene is considered to be a modifying gene. A special class of modifiers is the group that produce their effects by inhibiting the action of other genes. A commonly reported type of inhibitor is the flower color inhibiting factors.

Although varieties of plants may differ with respect to a given genetic factor, it is sometimes hazardous to make chemical or physiological comparisons among established varieties since genes other than the ones under study may contribute to the phenotype. Ideally, physiological characters should be studied on an isogenic background, that is, in plants that differ only with respect to the gene difference being investigated. In practice it often suffices to compare sibling mutant and normal plants. In cases where secondary effects of a gene are being studied, special genetic methods may be called for to exclude the possibility that some of the measured effects are caused by other genes. These methods include using separate occurrences of the same mutation, in which it is assumed that closely linked genes would not be simultaneously mutated in each occurrence; repeated backcrossing and reisolation of a mutant so that it and the normal approach isogenicity; and crossing the mutant onto diverse backgrounds to eliminate special effects by randomization of background.

The author agrees with Haldane (8) that genetic characters for physio-

XUM

logical work should be used only when they have been carried through at least three generations.

Some techniques of genetics can be useful for physiological studies. These include: (*a*) crossing to obtain special gene combinations, as for studying a gene dosage series, or for investigating gene interaction; and (*b*) the use of linked characters as markers to identify other genotypes at stages where the direct recognition is not possible. For example, a genetic character expressed in the seed or seedling which is closely linked with a mature plant character may be useful in pre-selecting certain plants; or, a factor for early development of color in maize endosperm can be used as a basis for selecting linked mutant and normal seeds from the same ear during developmental stages. A third technique is illustrated by maize in which there are chromosomal rearrangements called B-type translocations where a particular chromosome fails to undergo normal separation at the second microspore division in the pollen grain. When B-type translocation pollen containing appropriate genes is employed, seed can be obtained in which the endosperm and embryo are of opposite type, the one hyperploid, the other deficient. It is then possible to assess the roles of embryo and endosperm in the expression of certain seed characters. See Anderson (9, 10) for discussion and applications of chromosomal and genic techniques in physiological genetics.

Examples of Physiological Characteristics that are Genetically Determined

Light response.—Recent advances in the study of plant responses to light have been reviewed by Liverman (11) and Toole *et al.* (12).

Differences in maturity date because of a single gene or a small number of genes have been reported for many plants. In some cases these differences in earliness are clearly photoperiodically controlled; in others it is not obvious whether earliness results from a different photoperiodic response or from earlier photoinductive sensitivity.

Maize is day-neutral; therefore, floral initiation occurs at any time of the year. Schaffner (13) considers that maize floral development is affected by photoperiod since most varieties produce pistillate flowers in their staminate inflorescences when grown without supplementary illumination in the winter. Singleton (14) has described a short-day mutant called indeterminate growth (*id*). Galinat & Naylor (15) more critically studied inflorescence development in *id* plants, and Liverman (16) worked with a similar or identical mutation of independent origin utilizing controlled growth chambers. As usually grown in the United States out of doors, *id* plants are killed by frost before flowering, since the critical photoinductive daylength is 13 hr. (15). In segregating families grown under long-day conditions the normal plants flower while the *id* plants remain vegetative and can then be photoinduced by short days. If short-day photoinduction of *id* plants is followed by10.5 to 11.5 hour days, the staminate inflorescence develops normally; if the postinductive days are 11.5 to 12.5 hr., nonfertile flowers are produced; if the postinductive days are longer than 15 hr., the terminal inflorescences are

partly pistillate and proliferate numerous plantlets that can be rooted and serve as a means of vegetative propagation (15, 16). *Id* is thus a short-day mutant in which development of the inflorescence is especially sensitive to daylength.

Tobacco is day-neutral in its light response. Allard (17 referred to a recessive short-day mutant called mammoth, which is indeterminate during the long days of summer. Flowering of mammoth *Nicotiana tabacum* occurs on short days and is not dependent on temperature in the ordinary range. Lang (18) reports that the normal allele of mammoth is incompletely dominant. Steinberg (19) studied crosses from mammoth *N. tabacum* with *N. rustica* where the mammoth progeny were backcrossed to *N. rustica*. The derived mammoth *N. rustica* grew indeterminately during the summer and flowered irregularly in the greenhouse during the winter. It was discovered that these plants have a cold requirement and flower independently of daylength. Here it appears that the mammoth gene alternately conditions a cold or a short-day requirement for flowering, depending upon the genetic background. As the author points out, the mammoth gene in *N. rustica* needs to be retransferred to *N. tabacum* for an unambiguous interpretation.

Although ordinary rice is day-neutral, some varieties exhibit a short-day flowering response when tested under the proper daylength conditions. In the simpler cases among his intervariety crosses, Chandraratna (20) finds that the short-day form is inherited as a single dominant gene. The evidence indicates that in other crosses modifying genes are superimposed on the simple genic day length response.

Little & Kantor (21) find that a single recessive gene in the sweetpea determines early flowering. The early flowering form blooms on short days, the late flowering type requires long days.

A maize mutant called Corn grass (*Cg*) responds to daylength in an unusual way. Plants carrying the dominant *Cg* gene tiller profusely, have narrow leaves, and produce poorly defined ears and few staminate flowers when grown on long days (22, 23). The plants are hardly recognizable as maize. On short days, *Cg* plants have a greatly reduced number of tillers and resemble normal plants in appearance and inflorescence. *Cg* can be regarded as a short-day mutant in which long-day conditions prior to floral initiation cause abnormal inflorescence and vegetative growth.

Little, Kantor & Robinson (24) report that a recessive gene for earliness in marigolds makes the plants flower in 12 instead of 17 weeks during the summer, but both early and late flowering types bloom in 11 weeks during the short days of winter. Weiss (25) lists two gene pairs that affect earliness in soybeans. One conditions a dominant late-flowering tall type versus early-flowering short; the other is a dominant gene for earliness versus lateness with no marked influence on plant stature. In *Arabidopsis* Harer (26) finds that the late-flowering character is dominant, with the likelihood that different degrees of earliness are due to multiple alleles; furthermore, on short days there is evidence that modifying genes play a role in flowering (27).

Genetic control of the light requirement for seed germination has been

indicated for a few plants, but for the most part, demonstrations of clear segregations in successive generations are lacking. Howard & Lyon (28) and Howard (29) crossed species of *Nasturtium* and determined that the light requirement is dominant over light indifference. Honing (30) finds that the light requirement for germination of tobacco seed is dominant. Kugler (27) compared a race of *Arabidopsis thaliana* that requires light for seed germination with races where there is no light requirement. In the first generation hybrid the light requirement is recessive, but in the following generation the character is influenced maternally. In view of the excellent background of work on light promotion and inhibition of seed germination carried out by the Beltsville group (12), the genetics of seed dormancy should be a fertile field for investigation.

In tomato fruit, the recessive gene yellow (*y*) determines yellow versus colorless cuticle (31). Homozygous *y* plants have yellow cuticle if ripened in the light, almost colorless cuticle if ripened in the dark. This effect is most striking in genotypes lacking the red and orange flesh pigments, in which case the *y*, or the dark ripened *yy*, fruit is white (32). In their studies of the light coloring process in tomato cuticle, Piringer & Heinze (33) measured the action spectra for formation of the flavonoid pigment and found it to be identical with curves for the photoperiodic response as represented by inhibition of *Xanthium* floral initiation and the promotion of lettuce seed germination. The *y* gene determines whether the flavonoid pigment can be produced in response to light. There is no evidence to indicate whether the failure of pigment formation in the *y* fruits is attributable to the absence in the fruit cuticle of the photoperiodic pigment or to the failure of some link between the receptor pigment and flavonoid formation.

The maize mutant lazy, usually regarded as ageotroic, affects the light response of the plant. Shafer (34) discovered that lazy maize seedlings are initially phototropic and negatively geotropic. After a few days they become simultaneously ageotroic and aphototropic, suggesting that the bases for the two phenomena may be closely interrelated.

Chlorophyll deficiencies.—Chlorophyll deficiencies are probably the most frequently observed class of mutants in higher plants. As might be expected, no simple explanation suffices to describe the basis for these defects. There are four main types of chlorophyll-deficient mutants: (*a*) light green, deficient in amount of chlorophyll, (*b*) virescent, which are initially white, yellow, or light green, but develop green color, (*c*) yellow, in which chlorophyll is lacking but yellow pigments are present, and (*d*) albino, which lack both chlorophyll and carotenoid pigments.

Chlorophyll mutants usually are able to grow until endosperm or cotyledon reserves have been exhausted. This suggests the obvious possibility that chlorophyll-deficient lethal mutants, having a defective photosynthetic mechanism, are simply unable to synthesize carbohydrate. Indeed, Spoehr (35) and Gorham (36) have succeeded in culturing albino maize plants to maturity by supplying sucrose through the leaves, and Langridge (37) has grown an albino *Arabidopsis* mutant to maturity by supplying sugar through

XUM

the roots. That chlorophyll-deficient lethal mutants are not all merely deficient in carbohydrate synthesizing ability is indicated by three other albino mutants of *Arabidopsis* (37) which show no growth response to added sucrose; by another *Arabidopsis* mutant that will not grow on sucrose, but becomes normal if supplied with sucrose plus thiamine; and by a maize yellow seedling mutant in which droplets of sugar solution exude from the leaf tips (38). Nor are such mutants merely deficient in ability to absorb carbon dioxide, since Smith (39) found that albino and normal, dark and light grown maize seedlings absorbed carbon dioxide at approximately the same rate.

Eyster (40) determined that light grown albino and yellow mutants contained only about one-third to one-half as much catalase as light grown normals, whereas mutants and normals had approximately equal catalase activity if dark grown. In the studies of Seltmann (41) the respiration of normal and albino maize seedlings was equally sensitive to cyanide inhibition if dark grown, but only the normal seedlings were cyanide sensitive when light grown. He concludes that the respiration of light grown albino seedlings is primarily nonphosphorylative in character.

Koski & Smith (42) using maize, and Wallace & Schwartig (43) using sunflower, have reported mutants that form protochlorophyll in the dark and demonstrated that at low light intensities the protochlorophyll is converted to chlorophyll but is destroyed by strong illumination. It may be that in albino mutants which form chlorophyll at low light intensities the defect is attributable to the absence of a mechanism for preventing the photodestruction of chlorophyll.

Normal phototropic bending in albino mutants has led Bandurski & Galston (44) and Wallace & Schwartig (43) to conclude that carotenoids cannot be the receptive pigments in phototropism. Borthwick, Hendricks & Parker (45) measured the light inhibition of elongation of the second internode of normal and albino barley mutants. Both types are about equally sensitive to light and have the same action spectra.

Highkin (46) describes a nonlethal mutant of barley in which chlorophyll *b* is absent, but in which the same total amount of chlorophyll is formed. Whitaker (47) finds an altered ratio and total amount of chlorophyll *a* and *b* in a light green muskmelon mutant. Normal ratios of chlorophyll *a* and *b* have been more frequently recorded (48, 46).

The greening of virescent mutants has been observed to be more sensitive to temperature than normals. Little, Kantor & Robinson (24) report that the virescent phenotype of a marigold mutant is expressed only at low temperatures, and Phinney & Kay (49) find that dark grown virescent and normal maize seedlings green at the same rate between 16.5 and 25° C., but below 16.5° the virescents remain yellow.

A special case of greening in an albino plant is found in the recessive ghost (*gh*) mutant of tomato, where Mackinney, Rick & Jenkins (32) report that *gh* plants grafted with normal scions sometimes develop green shoots that bear fruit. In as much as the fruit on such branches is albino and the authors state that the character is not transmitted cytoplasmically, it can

be assumed that no hereditary transmission is involved. Thus, greening of the albino *gh* plants is attributable to some nongenetic graft transmissible factor or factors.

An unusual chlorophyll mutant is the recessive brown fruit color in peppers described by Smith (50). Here, failure of chlorophyll destruction in the homozygous recessive genotype causes the mature fruit to be brown or green, depending on the presence or absence of other factors for background color.

Von Euler & Hellstrom (51) isolated a methylindole from one albino maize mutant. Other albinos and normal did not contain the substance.

Premature germination of seeds.—Although germination of seed prematurely within the fruit, ear, or head, without dormancy occurs in a number of cultivated plants, its inheritance has been little studied except in maize. Robertson (52, 53) has investigated eight genetically different premature germinating or viviparous maize mutants. In three of them he established by the use of B-type chromosomal translocations that the viviparous character is a property of the embryo genotype and not dependent on the endosperm; thus the normal dormancy control must be located in the embryo. It is noteworthy that among the eight viviparous mutants, four contain no carotenoid, or a reduced amount, in the endosperm and produce albino seedlings, and another has no reduction of endosperm carotenoid but produces albino seedlings. In sunflower, seed of an albino mutant germinate prematurely (43). The frequency with which genetically viviparous mutants produce albino seedlings argues for a close functional relationship between the two phenomena. There is no evidence for an effect of light on the germination of viviparous seed, although the photoperiodic pigment role in the dormancy of lettuce, *Lepidium*, and other seeds (12) suggests the possibility.

Geotropic response.—A recessive ageotropic mutant in maize called lazy has been described by Van Overbeek (54, 55). For the first few days lazy seedlings display normal negative geotropic response, that is, reorient and grow upward if turned from the vertical. Older lazy plants are ageotropic and remain horizontal when blown over, so that they are typically seen growing prostrate along the ground. In horizontally held normal stems, about 60 per cent of the auxin is in the lower half of the stem, 40 per cent in the upper half; in lazy plants with horizontal stems about 55 per cent of the auxin is in the upper half, 45 per cent in the lower half. Shafer (34) concluded that the lazy phenotype is expressed because of interference with the normal gravity-determined distribution of auxin. A lazy mutant of rice which has similar growth characteristics is known (56).

Dwarfness.—Dwarf mutants have been regarded as deficient in some aspect of growth hormone physiology since the work of Van Overbeek (57, 58) who compared auxin relations in six dwarf maize mutants. In one mutant the tissues destroy more indoleacetic acid than normal tissues, and give off less auxin, as measured by diffusion into agar blocks. The other five dwarf mutants also produce less auxin. According to Harris & Phinney (59) the shorter coleoptiles of dwarf-1 maize have the same number of epidermal

XUM

cells as the normal, but respond only half as much by length growth to treatment with indoleacetic acid. The amount of auxin present in the tissues and the production of auxin is lower in dwarf-1 than in normal coleoptiles.

A differential response of dwarf and normal rice and peas to indoleacetic acid further accents the importance of auxin in the physiology of genetic dwarfs. Nakayama (60) finds that a concentration of indoleacetic acid which produces a 10 per cent inhibition of the normal causes a 19 per cent increase in the length of dwarf rice coleoptiles. The coleoptile tips of dwarf plants contain less auxin than normals. Von Abrams (61) obtained a 30 per cent increase in growth of etiolated dwarf peas by indoleacetic acid spray applied at a level which inhibits the tall variety. However, he found no difference between dwarf and normal plants with respect to: inactivation of indoleacetic acid by epicotyl sections or a filtrate of macerated epicotyl; diffusion of indoleacetic acid from excised apical buds; free auxin in the tissues; auxin released by alkaline hydrolysis; or tryptophan conversion to indoleacetic acid by epicotyl sections. Brian & Hemming (62) confirm the differential indoleacetic acid stimulation of growth in dwarf and normal peas.

Brian & Hemming (62) have reported a remarkable stimulation of dwarf varieties of peas (*Pisum*), broad bean (*Vicia*) and the French bean (*Phaseolus*) by gibberellic acid, a growth-regulating compound derived from a fungus of the genus *Gibberella*. By proper dosage of gibberellic acid, dwarf and normal plants grow equally tall. Phinney (63, 64) has demonstrated that gibberellic acid makes five of seven genetically different dwarf maize mutants grow normally. None of these mutants respond to indoleacetic acid, indoleacetonitrile, indolebutyric acid, indolepropionic acid, 2,4-dichlorophenoxyacetic acid, 2-naphthoxyacetic acid, or α-naphthaleneacetic acid. Furthermore, extracts of certain natural materials including young seed of maize, peas, lupin, and tobacco, contain substances that promote the normal growth of dwarf mutants in the same way as gibberellic acid (65).

Efficiency of inorganic element utilization.—It is commonly observed that varieties of cultivated plants differ in their tolerance of mineral element deficiency or toxicity in the soil. In a few cases such responses have been related to single gene differences. Weiss (66) observed that some varieties of soybeans develop iron-deficiency symptoms on calcareous soils, whereas other varieties grow normally. He determined that a single dominant gene, *Fe*, is responsible for efficient utilization of iron. In nutrient culture on low available iron, *Fe* plants appear green and healthy, but homozygous *fe* plants show severe iron chlorosis and eventually die. Homozygous, recessive, inefficient, iron-utilizing plants (*fe fe*) have in their stems a higher content of total iron, less soluble iron, less potassium, and extracts show a higher pH, than *Fe* plants. It is postulated that because of higher pH the iron in *Fe* plants becomes less available, thereby inducing iron deficiency.

Susceptibility to boron deficiency in celery, as indicated by the appearance of cracked stem disease, is determined by a single gene difference, according to the work of Pope & Munger (67). Segregating families can be classified by growing them on nutrient solution containing a deficient level

of 0.01 p.p.m. of boron. Susceptibility is inherited as a simple recessive. The same authors also found that sensitivity to magnesium deficiency in celery is determined by a single recessive gene (68). Genetically susceptible plants suffering from magnesium deficiency contain less magnesium in their leaves than normals, indicating that inefficient uptake is the basis for susceptibility, rather than inefficient utilization of an adequate amount of element, as was the case with inefficient iron-utilizing soy beans.

Cyanogenesis.—Cyanide produced by white clover arises from the enzymatic hydrolysis of the glucosides lotaustralin and linamarin, which respectively are glucose-HCN-methylethylketone, and glucose-HCN-dimethylketone (69). The hydrolytic enzyme in clover was found by Coop (70) to be almost identical in Michaelis constant, substrate specificity, and pH optimum with linamarase from linseed. Corkhill (71) determined that among a collection of clover plants some contain enzyme alone, some only glucoside, some both enzyme and glucoside, and some contain neither enzyme nor glucoside. Atwood & Sullivan (72) found that the presence of glucoside and enzyme are each determined by a single dominant gene. Differences in the cyanide reaction intensity suggest that there may be genetic modifiers of the amount of enzyme or substrate. An interesting feature of cyanogenesis in clover is the conclusion that has been drawn regarding the nonreversibility of glucoside synthesis and hydrolysis. In a plant that contains glycoside but no enzyme, the glucoside could hardly be synthesized by a reversal of the hydrolytic process since there is no enzyme present capable of carrying on the hydrolysis.

Disease resistance.—In the onion, red, yellow, and white bulb color are determined by three pairs of genes (73). Rieman (74) demonstrated that resistance to smudge, *Colletotrichum circinans*, is associated with red or yellow pigment in the outer scales of the bulb. In cases of intermediate coloring, the resistance is intermediate. Link, Angell & Walker (75) and Link & Walker (76) isolated protocatechuic acid and catechol from pigmented onion scales and showed that these substances parallel pigment and smudge resistance, and that they are effective as *in vitro* inhibitors of the smudge organism. Furthermore, colored portions of pigmented bulbs exposed to leaching by water or bleaching by sunlight contain less protocatechuic acid and are more susceptible to smudge infection than undamaged pigmented portions of the same bulbs.

Accumulation of ultraviolet fluorescing substances.—Seedlings of annual ryegrass, *Lolium multiflorum*, growing in contact with filter paper, frequently secrete something from the roots which causes the adjacent paper to fluoresce blue in ultraviolet light: the roots themselves do not fluoresce. Pure strains of perennial ryegrass, *L. perenne*, do not exhibit this fluorescence. Corkhill (77) and Linehan & Mercer (78) found that the ability to exude fluorescent materials from the root behaves as a simple dominant character. The fluorescent material has not been isolated.

Teas & Anderson (79), working with progeny from irradiated maize seed, discovered a recessive mutant in which the seedlings fluoresce blue in

XUM

ultraviolet light. The fluorescence is strong in young seedlings and then disappears, so that in a few weeks fluorescent and normal plants can no longer be distinguished; however, when the plants flower the mature anthers show fluorescence as a dominant character. Anthers of heterozygous plants contain about half as much fluorescent material as the homozygous mutant anthers. Paper chromatography revealed that the fluorescent material consists of three main components, all of which have biological activity as anthranilic acid in replacing tryptophan for *Lactobacillus arabinosus*. One of the blue-fluorescing substances has been isolated and identified as anthranilic acid. It is apparent that although anthranilic acid might be metabolically related to tryptophan, it cannot be accumulating at the expense of tryptophan, since it is present in the mutant at 10 to 30 times the molar equivalent of tryptophan.

Another seedling-recessive, anther-dominant blue fluorescent mutant in maize is described by Anderson (80). In this case chromatography shows that anthranilic acid and related substances are not involved.

Finkner *et al.* (81) have obtained evidence in oats that two dominant genes determine blue fluorescence in the seed. Each of the two gene pairs give simple 3:1 segregations when present alone, and the expected 15:1 ratios when both are involved.

Rubber versus triterpene synthesis.—Wildman *et al.* (82) studied the inheritance of rubber and resin in the hybrid and F_2 generation from an intercross between *Cryptostegia grandiflora* and *C. madagascariensis*. *C. grandiflora* contains 70 to 80 per cent rubber and less than 20 per cent acetone solubles in the dried latex; *C. madagascariensis* latex contains more than 80 per cent acetone solubles and less than 5 per cent rubber. The hybrid more nearly resembles *C. grandiflora* in having 60 to 65 per cent rubber and 25 to 35 per cent acetone solubles. Hendricks & Wildman (83) determined that the major constituent of the acetone soluble material is the triterpene alcohol, lupeol, which is present as long chain hydroxy-fatty acid esters. The F_2 generation derived from the hybrid consists of approximately three plants containing predominantly rubber in the latex to one containing predominantly lupeol esters. The evidence suggests that rubber versus triterpene formation is controlled by allelic genes. They postulate that a common precursor is converted alternatively to the linear isoprenoid rubber or to the cyclic isoprenoid lupeol, depending on the genotype of plant, as shown at the top of the next page. The recent finding by Teas & Bandurski (84) that rubber-bearing latex, at least in *Hevea brasilinesis*, contains enzymes capable of incorporating acetate into rubber, opens the way for a direct enzymatic investigation of the genetically conditioned difference in *Cryptostegia* latex isoprenoids.

Growth factor requirements.—The first organic growth factor-requiring mutants of higher plants have been produced and tested by Langridge (37). As experimental material he used a small crucifer, *Arabidopsis thaliana*, which is so small that 50 individuals can be brought to maturity in a single petri dish. It has the relatively short life cycle of 28 days, high fertility and

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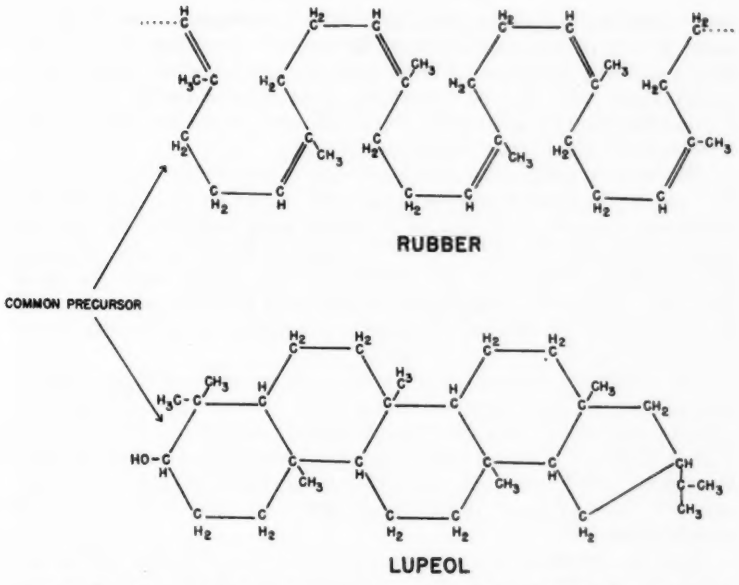


seed production, and a low chromosome number (n = 5) (85). Experimental plants were grown in sterile culture by adaptations of the *Neurospora* mutant production and screening techniques developed by Beadle & Tatum (86). Second generation progeny from irradiated seed were screened for mutants on a minimal inorganic medium solidified with agar. Nine mutants that were unable to grow on the minimal medium were obtained from 110 plants. One mutant requires thiamin; a second responded only to coconut milk among the supplements tested; another was albino and grew slowly and matured if supplied with sucrose; three others lacked chlorophyll but did not develop on sucrose; and the last three were green and did not grow beyond the two leaf stage with any supplement. This kind of mutant study should provide valuable information on the biosynthesis of growth factors in higher plants. The use of a diploid organism permits the continued propagation in the heterozygous state of difficult-to-culture lethal mutants. However, as the author admits, the number of cell generations in the formation of a seed undoubtedly provides a strong selection mechanism against many mutants such as ones that require substances which cannot diffuse into the seed fast enough to keep the cells alive. Therefore the range of mutants to be expected in higher plants is less than those found in microorganisms, where diffusion of essential factors is never a matter of more than a few microns.

Alkaloids or bitterness.—Bitterness or high alkaloid content in yellow and blue lupines is dominant over sweetness or absence of alkaloids. Von Hackbarth & Von Sengbusch (87) found that any of three genes produces the

sweet character in *Lupinus luteus*, and either of two genes gives rise to the sweet phenotype in *L. augustifolium*. Bitterness or sweetness shows a temporary maternal carryover; a bitter mother plant bears only bitter seeds, although any seeds that are genetically sweet outgrow the bitterness in a few months; likewise, genetically bitter seeds borne on a sweet plant require time to develop the bitter phenotype (88).

The toxicity of lupine alkaloids is sufficient to attach economic importance to sweet versus bitter lupines as forage (89). Rapid chemical tests for distinguishing between sweet and bitter lupines have been developed by Von Schwarze (90) and Wuttke (91). By the use of the latter test, approximately 10^6 individual plants have been classified during a single year. Forbes & Beck (92) have used a high local infestation with thrips for a novel and effective classification method. The insects accumulate on and damage sweet lupines but do not feed on bitter plants.

Genetically determined bitterness has been encountered in the cucurbit family. Barham (93) determined that a single dominant gene differentiates ordinary cucumbers (*Cucumis*) from very bitter tasting ones. On the gourd Pathak & Singh (94) found that bitter taste is determined by a single dominant gene, and Joubert (95) reported a dominant bitter watermelon mutant. The bitter principles in wild South African *Cucumis* species seem to be nonalkaloidal (96).

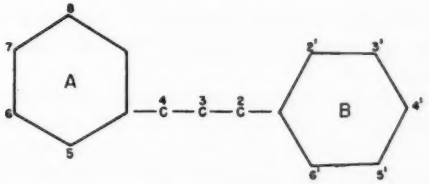
Lipides.—Members of a class of seedling mutants in maize called glossy are characterized by waxy-looking leaves, and are reliably classified by the fact that droplets of water adhere to glossy leaf surfaces, whereas few or no droplets remain on the normal leaves. Kurtz (97) determined that, in spite of the waxy appearance, glossy seedlings contain only about 70 per cent as much wax as normal, but approximately the same amount of fat. In most of the glossy mutants the fats are solid at room temperature, those of normals are liquid.

In cotton, white versus green lint differ by a single gene. Conrad & Neely (98) showed that in addition to the color difference, white lint cotton contains 0.5 to 0.6 per cent wax, and green lint cotton contains 12.6 to 15 per cent wax. The hybrid is intermediate, resembling the white parent more.

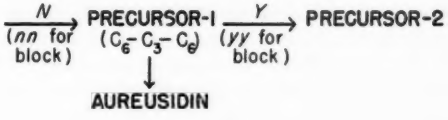
Endosperm mutants of maize in which large amounts of soluble carbohydrates accumulate have been found to contain more lipide than normal. Lindstrom & Gerhardt (99) recorded more lipide in sugary-1, Laughnan (100) found more in shrunken-2, and Teas & Teas (100a) reported higher lipide levels in brittle-2 endosperms. Laughnan has speculated that the increased lipide content of such mutants may be a consequence of the greater amount of sugars available as precursors.

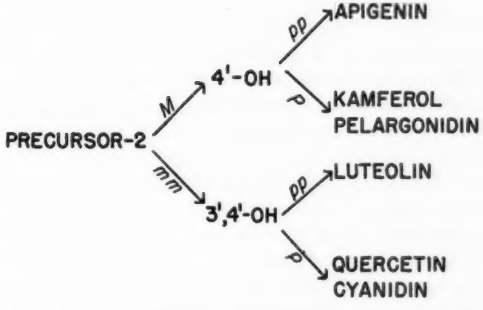
Flavonoid pigments.—The structure and natural occurrence of flavonoid compounds has been covered in an excellent review by Geissman & Hinreiner (101).

The chemical genetics of flavonoid compounds found in flower petals, leaves, and stems of plants has involved the following C_6-C_3-C_6 structure:



in which hydroxyl and methoxyl substitution of the A and B rings, the oxidation state and bond relations in the three carbon portion, and glycoside combinations, determine the type of pigment formed. The most complete study of flower pigment chemical genetics has been reported by Geissman and coworkers (102 to 105) from their work with snapdragons. They have investigated the roles of four genetic loci that are concerned with flavonoid pigments in the flowers: *P*, which determines the presence of anthocyanins and flavonols; *M*, which determines whether 4′-hydroxy or 3′,4′-dihydroxy derivatives of flavone, flavonol, and anthocyanin are present; *Y*, which affects the amount of aurone pigment; and *N*, which concerns the formation of the C_6-C_3-C_6 basic structure, probably either through controlling the synthesis of the A ring or the union of it with the appropriate B ring-C_3 moiety. They propose the following general pathway for flavonoid synthesis in the snapdragon:





Dayton (106) has considered pigment formation in several species of the snapdragon and concluded that the final products are the result of interac-

tion and competition between precursors. Earlier work on flavonoid pigments has been summarized recently by Dayton (106) and Geissman, Jorgensen & Johnson (102).

Storage carbohydrate.—The type and amount of carbohydrate stored in seeds has been shown to be affected by a number of genes. Bridel & Bourdouil (107) and Tanret (108) find that the recessive gene for wrinkled seed in peas, one of the genetic characters studied by Gregor Mendel, is associated with lower starch, increased sucrose, and an increase in the tetrasaccharide stachyose compared to the smooth seed form. In maize, sugary-1 is a recessive gene that resembles the wrinkled character in having less starch and larger amounts of soluble carbohydrates; sugary-1 has increased water-soluble polysaccharide and sucrose (109). In shrunken-2, another recessive gene of maize, the endosperm contains less starch and increased sucrose and reducing sugars. Laughnan (100) states that there is very little water-soluble polysaccharide accumulated in the shrunken-2 sugary-1 double mutant. This he interprets to mean that the shrunken-2 gene interrupts the conversion of small carbohydrate molecules to larger ones prior to the action of sugary-1.

Maize starch typically contains about 75 per cent amylopectin, a branched chain form of starch, and 25 per cent amylose, a straight chain form of starch (110). Cameron has analyzed a gene dosage series involving sugary-1 and another recessive, dull, where, because of genic interaction, he obtains amylose contents up to 65 per cent. Dunn, Kramer & Whistler (111) have made the triple homozygous combination of sugary-1, dull, and sugary-2, another gene similar to sugary-1 in action. They find that although the per cent starch is greatly reduced, the per cent amylose in the starch has been further increased to 77 per cent. Kramer, Whistler & Anderson (112) report that a gene for high amylose increases the amylose to 60 per cent without the increased water-soluble polysaccharide content and low starch-yield characteristic of high amylose lines obtained by sugary dull interaction.

The storage of 100 per cent of the starch in maize endosperm as amylopectin is controlled by waxy, a recessive gene (110). Brimhall, Sprague & Sass (113) describe a waxy allele from Argentina in which the starch is 98 per cent amylopectin. Waxy endosperm starches from rice, maize, sorghum, millet, and barley have been examined by Hixon & Sprague (114). The combination of the high amylose and waxy genes should be an interesting one for studying gene action, since the two factors alter the starch composition in opposite directions.

Cold requirement for flowering.—The cold or vernalization requirement for flowering of many biennial plants is well recognized. In sugar beets Abegg (115) finds that a single gene determines bolting (*B*) or annual versus biennial type (*bb*) flowering response. Normal and *B* plants both flower on long days; the *B* gene eliminates the requirement for winter dormancy. In *Hyoscyamus niger* a single incompletely dominant gene determines the biennial versus annual type (116). It is interesting in this regard that Lang (117) finds that gibberellic acid replaces the cold treatment for biennial

H. niger, but not the long-day light requirement. Gibberellic acid therefore takes over the role of "vernalin" but not "florigen."

Fragrance.—Although varieties of plants differ in flower fragrance or volatile essential oils, there are few examples of genetic determination of odor or fragrance. According to McLean (118) a violet-like fragrance is dominant in the sweetglad, with two genes being required for the expression. Also, a jasmine-like odor in sweetglads is determined by two factors; if one is homozygous, the other acts as a dominant. In rice, Jodon (119) reports a violet-like odor of the flowers that is attributable to a single recessive gene.

Carotenoids.—The inheritance of carotenoids has been studied most extensively in the series of genes that determine fruit color in the tomato. Red tomato fruit, in which the carotenoid is lycopene, owe their color to the dominant gene *R* (120). Three recessive genes markedly reduce the amount of all carotenoids. These are, in increasing order of effectiveness: apricot; *r*, the recessive allele of red; and ghost (*gh*) (32, 121). Homozygous *gh* plants are albino, lacking chlorophyll as well as carotenoid pigments and must usually be grown as grafts with normal plants. Two other genes determine the type rather than the amount of carotenoid that is formed in the fruit. They are: the recessive gene tangerine associated with reduced amount of lycopene, but having major quantities of prolycopene and *zeta*-carotene (122); and the dominant gene beta (*B*), which is associated with the carotenoid being predominantly *beta*-carotene (123). Tomes and coworkers (123, 124) have studied genotype interactions and reported the action of a dominant gene that modifies the expression of *B* by reducing the *beta*-carotene levels.

Evidence for the early precursor relationships in the biosynthesis of carotenoids are based for the most part on work with other organisms. The most thoroughly studied case of isoprenoid biogenesis involves the steps in cholesterol formation in animal tissue. However, in plants, Arreguin, Bonner & Wood (125) working with guayule, find that the isoprenoid rubber can be formed at the sole expense of acetate, and Bonner (126) reports that the five carbon acid, *beta*-methylcrotonic, is a more efficient source of rubber than is acetate. Evidence for acetate as a precursor of carotenoids comes from the fungus *Mucor hiemalis*, where Grob & Butler (127) have fed carboxyl- and methyl-C^{14} labeled acetate in parallel experiments and partially degraded the isolated *beta*-carotene. They find that the distribution of label is consistent with the origin of *beta*-carotene through an acetate-involved four, six and five carbon acid series, as postulated by Bonner (126) for terpene origin. Zabin (128), in a preliminary account, reports that radiocarbon-labeled acetate is incorporated into lycopene in ripening tomato fruit.

It seems likely that early steps in the formation of tomato carotenoids occur by pathways similar to those outlined for rubber and cholesterol. The tomato fruit carotenoid pigments could be derived from a basic C_{40} unit by dehydrogenation, cyclization, and isomerization. The evidence is not clear with regard to the roles of genes in later steps. Lincoln & Porter (121) have attempted to arrange the fruit color genes as blocks in sequential reactions, whereas Jenkins & Mackinney (122) favor the view that there is little or no

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conversion of many of the carotenoids one to another, but rather that parallel syntheses are involved.

In maize the gene yellow (*Y*) quantitatively determines the level of vitamin A active carotenoid pigment in the seed. It has been shown that total carotenoid increases directly with gene dosage, but that the ratio of four vitamin A active pigments remain constant for the differences of 1.8, 4.2, and 6.4 p.p.m. of total carotenoids found in seed having respectively one, two and three doses of *Y* in the endosperm (129, 130).

B-vitamin content.—The effect of the sugary-1 gene on endosperm niacin levels in maize has been the basis of several studies since the reports of Barton-Wright (131) and Burkholder, McVeigh & Moyer (132) that sugary-1 maize kernels contain about twice as much niacin as those of normal maize. The relationship between sugary-1 and high niacin was established by analyses of endosperms from three new radiation-induced occurrences of sugary mutations (133), and by Cameron & Teas (134) who found that the niacin content of maize endosperm varied with gene dosage in a sugary-1, dull series of kernel genotypes. Analysis of developmental stages of sugary-1 and normal kernels established that niacin was higher in sugary-1 from early development (135). Furthermore, it has been established that the niacin level of endosperm tissue is determined by the endosperm genotype and is independent of embryo genotype (136). The localization of high niacin content in the aleurone layer of the endosperm has been demonstrated in dissected kernel fractions (137, 138).

The higher level of niacin in sugary-1 endosperm is not specific for that vitamin. Teas (133) has reported assays of a diverse series of sugary-1 and normal endosperms for niacin, choline, pyridoxine, riboflavin, inositol, biotin, pantothenic acid, and thiamine. All eight B vitamins were higher on a per cent basis in sugary-1 than normal, indicating the need for a general explanation of higher vitamin level, rather than a special role for niacin.

Six morphological endosperm mutants other than sugary-1 contain higher niacin on a percentage basis than did comparable normal kernels (139, 140). Thus, higher niacin seems to be a common feature in mutants that affect the appearance of the maize endosperm.

Miscellaneous.—Griffith, Valleau & Stokes (141) have studied a widely distributed dominant gene associated with the property of converting nicotine to nornicotine in tobacco. This demethylation process is brought about by a thermolabile factor, presumably enzymatic. Schrock (142) reports that a single recessive gene determines low coumarin content in *Melilotus*. Single gene differences have been found to cause pod shattering or fruit dehiscence in soybeans (25), lupine (143), sesame (144), lettuce (145), rice (146), and pepper [*Capsicum* (147)]. White (148) reviews the genetics of fasciation. Kehr (149) discussed genetic tumors in tobacco species crosses.

Conclusions

It can be anticipated that physiological genetics of higher plants will advance by application of methods and techniques developed for physiological genetic studies in microorganisms. One of the most valuable is the

method of studying a series of mutants of the same class, analogous to the study of a group of methionine-requiring mutants of microorganisms for determining the physiological relations of methionine. In higher plants, suitable groups of mutants that occur frequently such as chlorophyll deficient, dwarf, and anthocyanin types are often available in the stocks of geneticists particularly where radiation mutation studies have been carried out. Alternatively, mutants can be obtained by screening natural populations or mutagen-treated progeny. The use of rapid or automatic screening systems like the rapid lupine chemical test (91) or the method of selecting pathogen resistant mutants in oats as developed by Wheeler & Luke (150) make selection of single individuals among 1×10^5 to 1×10^7 feasible. Special detection methods could be devised for many other types of mutations.

In view of the complications that attend gene changes by way of altered metabolic patterns, there is need to analyze many cases of single gene effects and gene interactions before effectively attacking such problems as the physiological basis for oil content in maize, where Sprague & Brimhall (151) have evidence that 20 or more genes are involved, or the more complex aspects of plant growth like yield in agronomic plants.

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XUM

EFFECTS OF ANTIBIOTICS ON PLANTS[1]

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Introduction

In recent years increasing attention has been paid to the effects of antibiotics on higher plants. There are several reasons for this new interest; five of these are outlined here with references to appropriate recent reviews. (*a*) It now seems likely that some antibiotics may be produced naturally in soil in the vicinity of particles of organic matter or in the rhizosphere; consequently roots may be naturally exposed to their influence and, indeed, other tissues also if the antibiotics are taken up by the roots and translocated (1 to 4). (*b*) Many phytopathogenic fungi and bacteria produce phytotoxic metabolic products in artificial culture and in some cases also *in vivo* (5 to 8); quite a number of these toxins are also antibiotics in so far as they are also toxic to some microorganisms. (*c*) Many antibiotics have been isolated from tissues of higher plants (9, 10); their natural significance is uncertain; it may be that they are concerned with protection against microbial parasites in some cases (11), but, in so far as many of them are also inhibitors of plant growth, they may be involved in growth regulation or phenomena of dormancy. (*d*) The discovery that some antibiotics can be used for control of bacterial and fungal diseases of plants and that some are systemic in effect (12, 13, 14) has inevitably led to studies of phytotoxicity, translocation and metabolic modification of systemically distributed antibiotics in plant tissues. (*e*) The unique structure and biological activity of antibiotics has naturally led to a good deal of empirical observation of the effects of antibiotics on plant growth and metabolism and, as we shall see, remarkable instances of inhibition and stimulation of growth have been recorded. It will be seen that of the five reasons for botanical interest in antibiotics listed above, three (*a*, *b*, and *c*) derive from a belief that plants may be affected by antibiotics under natural conditions, and two (*d* and *e*) derive from a belief that practical advantages may be obtained from the application of antibiotics to plants.

It might be expected that because of these several very different methods of approach it would prove difficult to review the results of research in any unified manner. Be that as it may, whatever the reasons for initiating researches, the fact is that most of the experimental work so far reported has been concerned with the effects on plants of exogenously applied antibiotics and little work has been reported bearing directly on possible natural circumstances in which antibiotics affect plants. The present review, therefore, is mainly devoted to collecting together the somewhat scattered information

[1] The survey of the literature pertaining to this review was completed in March, 1956.

XUM

on the inhibitory or stimulating effects of exogenously applied antibiotics on plant metabolism and on their translocation in plants. Unfortunately, the novelty of the field and the anecdotal nature of many reported observations preclude a really critical treatment. Consideration has been restricted to pure antibiotics. A mass of information is, for instance, available on phytotoxic properties of culture media on which fungi or bacteria have been grown; experience has shown that it is quite impossible to interpret such observations without fractionation of the culture filtrates, as observed effects may be due to toxic compounds of relatively low molecular weight, to the activity of enzymes, especially the pectic enzymes, or to the purely physical effects of such macromolecular substances as polysaccharides or proteins.

Nomenclature of antibiotics.—Several well-known antibiotics are known under different names. Names used in this review, with synonyms in parenthesis, are: actidione (cycloheximide); bacillomycin A (fungocin); chloramphenicol (chloromycetin); chlortetracycline (aureomycin); oxytetracycline (terramycin); patulin (clavacin, clavatin, expansine).

Inhibitory Effects

Table I summarises very briefly the recorded observations of phytotoxic effects of antibiotics. A commonly used test of phytotoxicity has been to germinate seeds in antibiotic solutions or to make observations on the growth of young seedlings transferred to such solutions. Such tests have given results listed in columns B, C, and D of Table I. Solutions of antibiotics sprayed on foliage of plants sometimes cause foliage lesions; observations of this kind have frequently been made in the course of work on the use of antibiotics for plant disease control (column E). Cut shoots of plants placed in antibiotic solution may develop toxic symptoms, such as loss of turgor or stem and leaf lesions; references to observations of this kind are given in column F of Table I.

Most antibiotics that have been examined thoroughly have been found to be phytotoxic to some extent. By far the least toxic is penicillin (in addition to references given in Table I, see 36, 37, 52, 65, 69, 70, 71) and some of the recorded cases of toxicity may well have been due to impurities. Bustinza & Lopez (52), for instance, found that pure penicillin was not inhibitory to radish or lettuce seedlings; they attributed the phytotoxicity of some impure samples of penicillin to contamination with indoleacetic acid and phenylacetic acid. Even some of the more definitely toxic antibiotics, such as griseofulvin, chloramphenicol, and streptomycin, can nevertheless be distributed systemically in plants in significant concentrations without visibly affecting plant growth or metabolism. Some of the more interesting inhibitory effects are mentioned below.

Inhibition of germination and seedling growth.—Four antibiotics are of interest in so far as they consistently inhibit seed germination or root growth at concentrations of 5 μg./ml. or less; these are actidione, azaserine, alter-

TABLE I

SUMMARY OF TOXIC EFFECTS OF ANTIBIOTICS ON HIGHER PLANTS*

(A) Antibiotic	(B) Inhibition of germination	(C) Inhibition of root growth	(D) Inhibition of shoot growth	(E) Foliage toxicity	(F) Wilt toxin
Acidione	15 to 18	19		15, 20, 21	
"Agrimycin"				22, 23	
Albidin	24	24			
Alternaric acid	24,25,26	24,25,26		25 to 29	25 to 31
Alternarine	32	32	29, 32		
Antimycin				33	
Azaserine		34			
Bacillomycin	35				
Bacillomycin A	35				
Bactracin		36			
Catenulin		36			
Chloramphenicol (=Chloromycetin)		36 to 39	39		
Chlortetracycline (=Aureomycin)		36 to 39	39		
Citrinin	17, 24	24, 41			
Filipin	40				
Fusarinic acid				42	43
Gladiolic acid	17, 24	24			
Gliotoxin	17, 24	24			
Glutinosin	24	24			
Gramicidin	44		44		
Griseofulvin	24	1, 24, 45	1, 44, 45	1, 44	
Helixin	46, 47	47			47
Humulon	17				
Lupulon	17				
Lycomarasmin					30, 48, 49, 50, 51
Mycophenolic acid	24	24			
Neomycin		38, 39	39		
Oxytetracycline (=Terramycin)		36 to 39, 41	39, 41		
Patulin (=Clavacin)	17, 24, 52, 53	24, 41, 54	41, 55	26, 55	49, 50
Penicillin		24	44		
Polymyxin		36, 56			
Pyocyanine	44		44		
Rimocidin		36			
Stemphylone	57	57			
Streptomycin	16, 24, 58, 59	24, 36, 37, 38, 39, 59, 60, 61, 62, 63, 64	39, 44, 59, 61, 62, 63, 65, 66, 67, 68	22, 23	
Thiolutin		36			
Viridin	17, 24	24			
(Antibiotic)XG	17				

* The numbers refer to entries under "Literature Cited."

naric acid, and polymyxin. Actidione is highly phytotoxic. It reduces root growth of peas at 1 μg./ml. [Wallen *et al.* (17, 19)]. Though it is also toxic to foliage there seems to be some specificity in its action since germinated seeds treated with actidione have been reported (19) to show roots of reduced size but epicotyls much longer and stouter than in untreated plants.

XUM

A more marked specificity is shown by azaserine (*o*-diazoacetyl-L-serine) which, though inhibitory to root growth of several species in the range 1 to 10 μg./ml., has very little effect on shoot growth even in quite large doses (34). Alternaric acid is about as inhibitory as actidione to root growth (26) but this is associated with very marked toxicity to shoot tissues (see below).

Polymyxin is a polypeptide antibiotic produced by some strains of *Bacillus polymyxa*. It has been shown by Norman (56) to inhibit root growth of barley and other species in the range of 5 to 10 μg./ml. and the effect of doses in this range can be reversed by Ca^{++}. Apparently polymyxin disorganises the cell membranes of surface cells of the root, possibly as a result of its marked surface activity, and cell contents leak out. Its antibacterial properties are also due to a similar cell-wall eroding activity. Other polypeptide antibiotics, produced by aerobic spore-forming bacilli, with root growth inhibiting properties, are (Table I): bacillomycin, bacillomycin A, gramicidin, and antibiotic XG. It would be interesting to compare more closely a selection of polypeptide antibiotics of this type. The root growth inhibition produced by such substances is of particular interest in so far as the aerobic spore-formers are typical organisms of the rhizosphere and root surface. Root growth inhibition produced by streptomycin, much less potent in this connection than polymyxin, is also reversed by Ca^{++} and, even more effectively, by Mn^{++}. Its antibacterial activity is not reversible in this way, so that presumably inhibition of root growth and inhibition of bacterial metabolism are brought about by quite distinct mechanisms [Gray (64)].

Phytotoxic effects of antibiotics produced by parasitic microorganisms within plant tissues.—Much work has been published suggesting that some plant disease symptoms develop as a result of the release of toxic substances by the invading parasite. Few of these substances have been purified and characterized. The general subject is outside the scope of this review, and has been recently summarized by Dimond (6) and Brian (7), but a few cases, where toxins have been characterized and where they also possess antibiotic properties, can be appropriately considered here.

The pioneer work in this field is that of Gäumann and his colleagues at Zurich (30, 43, 48 to 51) on *Fusarium* wilt of tomatoes. They have sought to explain some aspects of the wilt syndrome by the production of toxins *in vivo* which, for the purposes of study, they isolated from *in vitro* cultures of *Fusarium lycopersici*. Two of these toxins are of known chemical structure; these are lycomarasmin, a peptide formed from asparagine, glycine, and α-hydroxyalanine (72), and 5-*n*-butylpicolinic acid (fusarinic acid) (73). Both possess antibacterial and antifungal properties (74). If cut shoots of tomato are placed in solutions of lycomarasmin, they develop symptoms of transpiratory unbalance leading eventually to wilt; under similar circumstances fusarinic acid causes leaf and stem lesions as well as eventual wilting. Recent American work (see references 6 and 7 for further information) has thrown some doubt on the significance of this work, for two main reasons:

(*a*) lack of evidence that these toxins are produced in the plant, and (*b*) the toxins alone do not reproduce such important features of *Fusarium* wilt as vascular occlusion and browning. At present the problem is by no means cleared up and more recent work (75) suggests that fusarinic acid, at least, is produced *in vivo*.

Alternaric acid is a metabolic product of *Alternia solani*, a pathogen of potato and tomato (76). The disease is characterized by the production of black lesions on stem and foliage of infected plants and in many of these lesions no fungal infection can be traced. The observation that alternaric acid will produce similar lesions led to the suggestion by Brian *et al.* (25) and Pound & Stahmann (28) that it may also be produced *in vivo*, causing lesions in advance of the fungus as a result of translocation. Unequivocal proof of this is still lacking and it appears likely that other toxins are also involved. Direct comparison of the effects of alternaric acid and of lycomarasmin on the water balance of cut shoots (26, 30) has shown that alternaric acid is by far the more toxic of the two.

The well-known antibiotic patulin is produced in apple fruit tissues infected with *Penicillium expansum* (77) but, in spite of its well-established phytotoxicity (see Table I) its presence appears to have little connection with the development of disease symptoms. It has been compared with lycomarasmin and alternaric acid by the cut shoot technique and produces symptoms quite distinct from either. Whereas alternaric acid and lycomarasmin produce lesions in intervenal areas of the leaves, patulin produces a very marked browning of the xylem of the stem and leaf veins [Gäumann *et al.* (49, 50); Brian *et al.* (26)].

Finally, mention may be made of the toxin produced by *Pseudomonas tabaci*, the causal organism of tobacco wildfire. This toxin, in addition to being toxic to higher plants, also inhibits growth of the unicellular alga *Chlorella*, and so may be considered to be an antibiotic in the usual restricted sense of the term; there is no published information on its toxicity to other kinds of microorganism. Most, but not all strains of the pathogen, when they infect tobacco leaves produce wide chlorotic halos around the point of infection. In a most elegant series of researches, Braun (78, 79, 80) has shown that strains which produce these halos also produce in culture media a toxin capable of reproducing these symptoms if applied to tobacco leaves. He was able to show that the toxicity of this substance to *Chlorella* was reversed by L-methionine and, in addition, that a known methionine antagonist, methionine sulphoxime, will produce halos in tobacco leaves similar to those caused by the toxin. He postulated, therefore, that the toxin acts in the tobacco leaf by competitive antagonism of some metabolic process involving methionine. This conclusion was strikingly supported by the work of Woolley *et al.* (81, 82, 83) on the chemistry of the toxin itself. Though it is very unstable they were able to isolate it in pure crystalline form and showed that it was itself structurally related to methionine. As Woolley (84) has

XUM

pointed out, we have here an interesting "example of the production (by a parasite) of an antimetabolite specifically directed against an important participating molecule in the cell of the host."

Inhibition of pigment production.—In a series of papers von Euler *et al.* (61, 62, 85) showed that if seeds of several species of plants were sown on blotting paper moistened with streptomycin solutions, the resulting seedlings were partially or completely devoid of chlorophyll. Since then this bleaching effect of streptomycin has been observed on several occasions (22, 23, 24, 38, 63, 67, 68, 86 to 90). The evidence is to some extent conflicting, but it appears to be fairly certain (*a*) that chloroplasts already formed and containing chlorophyll are not usually affected by streptomycin; (*b*) that unpigmented chloroplasts in etiolated plants do not develop chlorophyll in the presence of streptomycin when the plants are transferred to light; (*c*) that the effect of streptomycin is complex, since in its presence morphologically normal plastids without chlorophyll may be formed or, particularly in high concentrations, the plastids themselves may be interfered with, their number per cell being reduced and those formed reduced in size. The mechanism of the action remains quite unknown. Inhibition of chlorophyll synthesis by streptomycin has also been observed in carrot tissue culture [de Ropp (91)].

A similar effect was described in phytoflagellates by Provasoli *et al.* (92) and Jirovec (93), and the numerous subsequent observations have been reviewed by Hutner & Provasoli (94, 95). Exposure of some strains of *Euglena gracilis* to streptomycin leads to fragmentation and loss of chloroplasts with consequent loss of chlorophyll and photosynthetic capabilities; the carotene-pigmented eyespot is sometimes lost as well. The resulting apoplastidic organisms will continue to grow and reproduce indefinitely as saprophytes. In this latter respect *E. gracilis* is somewhat exceptional since in other species, such as *E. mesnilii* and *Chlorogonium euchlorum*, the induced colourless organisms are no longer viable. Streptomycin is only effective when the cells exposed to it are reproducing vigorously [Pringsheim & Pringsheim (96)]. Several workers have recorded that streptomycin is more effective in producing apoplastidic forms in the dark, and have supposed that chlorophyll has a protective effect on the plastid (58, 97), but the suggestion of Provasoli *et al.* (92) that light favours photosynthetic individuals is probably more correct.

Many other antibiotics have been tested but all have failed to show a similar effect on *E. gracilis* (94, 98). However, several have been reported to induce chlorosis in higher plants, including chlortetracycline (38, 39, 89, 99), oxytetracycline (38, 39, 87, 99), chloramphenicol (38), and alternaric acid (24). Tetronic acid derivatives have been reported to cause albinism in seedlings of several species of plant; of these 3-(α-iminoethyl)-5-methyltetronic acid is far more active than streptomycin in inhibiting chlorophyll formation in pea seedlings but is without effect on *E. gracilis* (100, 101). It differs from streptomycin in its mode of action in so far as chlorophyll is destroyed in leaves already formed at the time of treatment.

XUM

Synthesis of pigments other than chlorophyll may be affected. Pramer & Wright (38) have reported that anthocyanin is not formed in mustard seedlings treated with chloramphenicol; similar effects have been produced with methionine and even more readily with ethionine (102). This latter result recalls the effects of the toxin of *P. tabaci* discussed in a previous section of this review. On the other hand Wright (24) observed that while streptomycin reduced the chlorophyll content of radish seedlings, the amount of anthocyanin in hypocotyl and cotyledons was much increased. Oxytetracycline has a similar effect on radish; seeds soaked in a 100 μg./ml. solution produced seedlings with 8 per cent of the acetone soluble pigments (mainly chlorophyll) found in untreated plants but with 227 per cent of the content of anthocyanins [Nétien & Lacharme (87)].

Streptomycin and some other substances also affect carotenogenesis. The loss of the carotene eyespot in *Euglena* in the presence of streptomycin has already been mentioned. Inhibition of carotenogenesis in *Phycomyces* by streptomycin has been reported (103, 104), but this effect appears to depend on nutrient conditions because on some media streptomycin appears actually to increase carotenogenesis (103). Other substances reported to inhibit carotenogenesis in *Phycomyces* (104) are chloramphenicol, actidione, penicillic acid, and terrestric acid. The latter two substances, both fungal metabolic products, are tetronic acid derivatives but 3-(α-iminoethyl)-5-methyltetronic acid (see above) has no effect on carotenogenesis in *Phycomyces* (101). Streptomycin, chloramphenicol, and 3-(α-iminoethyl)-5-methyltetronic acid inhibit carotenogenesis in pea and wheat seedlings (67, 101, 103).

The inhibition of both chlorophyll and carotene synthesis by streptomycin and other substances is interesting. It may be purely coincidental, but it seems possible that the explanation is that the carotenoids and the phytol residue of chlorophyll have a similar biogenetic origin, as has been previously suggested for other reasons (105).

Growth Stimulation

A number of cases of plant growth stimulation by antibiotics have been reported, notably by Nickell (69, 70, 106). He found that certain antibiotics stimulated growth of *Rumex acetosa* virus tumour tissue, accelerated germination of some seeds, accelerated growth (measured by fresh weight) of *Lemna minor* in aseptic culture, and accelerated growth of seedlings of several species if antibiotic solutions were watered on to the soil where the seeds were sown.

Tissue culture.—The effects observed by Nickell were very marked. For instance, in an experiment where the "growth value" of *Rumex* tissue was 1.3, in the presence of 5 μg./ml. bacitracin the value was 10.9; somewhat smaller increases were produced by similar concentrations of penicillin, oxytetracycline, streptomycin, and thiolutin. Other workers (98, 100) have usually reported that antibiotics inhibit growth of tissue cultures, except

XUM

on occasion with some samples of penicillin (71, 107 to 111). De Ropp (71) has attributed the stimulating effect of penicillin to contamination of the antibiotic with indoleacetic acid. On the other hand, Camus & Lance (111) have obtained consistent stimulation of artichoke tuber tissue cultures with apparently pure penicillin G and procaine penicillin; however, the fact that they only obtained stimulation with auxin-requiring tissues and that the antibotic had no effect in the presence of indoleacetic acid strongly suggests that either penicillin replaces indoleacetic acid, which seems unlikely, or that the samples of penicillin were in fact contaminated with traces of auxin.

Plant growth.—It is perhaps best to consider first work on *Lemna* in aseptic liquid cultures (106) as this is a fairly simple biological system. A large number of antibiotics were tested at several concentrations. After three weeks' growth, some concentrations of bacitracin, penicillin G, streptomycin, and oxytetracycline had produced statistically significant, and in some cases quite large, increases in fresh weight; other antibiotics were toxic. After eight weeks' growth even such phytotoxic substances as citrinin, patulin and thiolutin had produced remarkable increases in fresh weight. Turning next to seedlings of various species growing in soil, Nickell recorded (69, 70) marked acceleration of seedling development following treatment of the soil with very low concentrations of penicillin and oxytetracycline. Essentially similar results have been reported by other workers (65, 66, 112, 113, 114).

Since the main common characteristic of these various antibiotics which have stimulated plant growth is their antibacterial activity, it is natural to suspect that a microbiological factor is concerned in the production of the observed effects. However, if the conditions in tissue culture and *Lemna* culture were in fact aseptic, such an explanation cannot be invoked to explain the results with those systems. In the work on seedlings in soil a microbiological factor may well be involved. Two pieces of evidence support this view: (*a*) Hervey (115) has shown that application of antibiotics to soil accelerates microbial growth and activity, and (*b*) Barton & MacNab (36) failed to demonstrate any stimulation by antibiotics of wheat seedling growth in culture solutions; indeed, most were toxic. They did, in fact, find that oxytetracycline, penicillin G, and thiolutin improved growth in one batch of distilled water. Growth in this water alone was poor and they obtained evidence that the stimulatory effect of oxytetracycline was attributable to its chelating properties and that a known chelating agent, ethylenediaminetetraacetic acid, also improved growth. Nevertheless, it is difficult to see how the activity of penicillin G could be explained on this basis, nor does it seem conceivable that a metal bonding effect could explain the activity of low concentrations of antibiotics in soil. Another possibility is that some antibiotics at least could accelerate growth by interfering with auxin metabolism (41). It has also been suggested that antibiotics may affect the rate of photosynthesis; Havinga *et al.* (116) demonstrated increases in carbon dioxide fixation in light by *Scenedesmus obliquus* in the presence of oxytetracycline and chlortetracycline, the latter greatly increasing the yield of su-

XUM

crose among the products of photosynthesis. Unfortunately, their results are difficult to interpret as equally great effects were on occasion obtained with simple buffer solutions. At present there is insufficient evidence to warrant further speculation concerning the mechanism of these stimulations; what is required is further experimental work under more closely defined conditions.

It is perhaps appropriate to mention here two fungal metabolic products which are not antibiotics and hence strictly outside the scope of this review. Gibberellin A and gibberellic acid, metabolic products of *Giberella fujikuroi*, do produce undoubted acceleration of growth of shoots of several species of plant whether growing in water culture or soil (117 to 125). Their mode of action has not yet been established; they do not appear to be auxins. These substances are treated more fully in the chapter by Stowe and Yamaki in this Volume (see p. 181).

Translocation of Antibiotics in Plants

Since Anderson & Nienow (60) in 1947 produced strong evidence that streptomycin could be taken up by the roots of soybean plants and translocated to the leaves, many similar observations have been made and it is now known that many antibiotics can be taken up and translocated. The various observations have recently been summarized by Crowdy & Pramer (14) and there is no need to do so again here. The following antibiotics are translocated in some plants: chloramphenicol, chlortetracycline, griseofulvin, mycetin, neomycin, oxytetracycline, penicillin, pleocidin, streptomycin, streptothricin, and viomycin (14, 126).

Most work has until very recently been at best of a semiquantitative nature, but certain facts interesting to plant physiologists have emerged. Quite large molecules (with molecular weights of the order of 200 to 500) are readily and rapidly taken up by the roots. Not all antibiotics are taken up and translocated and, not surprisingly, among those which are not included are such polypeptides as subtilin and gramicidin (14). Of those that are taken up some seem to move freely in any species of plant whereas others move in some plants but not in others. Crowdy & Pramer have pointed out that most of the readily translocatable antibiotics are neutral or acidic substances, whereas anomalous results are often encountered with basic or amphoteric compounds.

More recently Crowdy *et al.* (127, 128) have published strictly quantitative data on uptake of griseofulvin through roots of broad bean (*Vicia*) and tomato and on translocation to the shoots. The behaviour of this antibiotic in plants is better understood than that of any other. It would be dangerous to assume that all antibiotics introduced systemically will behave in exactly the same way, but some of the observations are probably of general importance. Crowdy *et al.* first established that griseofulvin was taken up and translocated unchanged in short term experiments (three days). Using a countercurrent distribution technique they succeeded in isolating griseofulvin quantitatively from plant tissues; for instance in a three-day experi-

XUM

ment, of 123 mg. griseofulvin supplied to the bean plants in the external solution, 82 mg. remained in the solution, 20 mg. were found in the roots, and 11 mg. in the shoots, leaving only 10 mg. unaccounted for. These data were used to confirm the accuracy of a method based on a bioassay, which was far more convenient to handle. Using the bioassay technique they were then able to show that the uptake and translocation of the antibiotic involves at least two processes. From a quantitative point of view the main process appears to be a passive movement of the compound through the roots and into the shoots with the transpiration stream, so that the amount taken up is linearly related to the amount of water transpired. This relation between transpiration and uptake had been demonstrated in general terms by Stokes (45). There is also a more rapid and somewhat more complex process of absorption by the roots. Within an hour of treatment an equilibrium is reached between the concentration of griseofulvin in the external solutions and that in the root tissues. Thereafter there is a linear increase in concentration with time, so that griseofulvin concentrations in the root reach higher values than in the external solution, probably as a result of solution in the root lipides. This process is independent of transpiration and takes place in detached roots. It is inhibited by sodium azide and dinitrophenol in concentrations below that necessary to affect the transpiration rate, whereas the first process, linked with transpiration, is not so inhibited. It should be added that there is no definite evidence that uptake of griseofulvin is an active process. Crowdy points out the similarity of this complex pattern of uptake with that of calcium and chloride ions reported by Hylmö (129).

Earlier work on systemic treatment of plants with antibiotics had suggested strongly that some breakdown takes place in the plant tissues, an observation of considerable practical importance. Crowdy *et al.* were able to put this on a quantitative basis in the case of griseofulvin in broad beans; a logarithmic "decay" of griseofulvin in the plant was demonstrated, and under the conditions of their experiments griseofulvin had a "half-life" of about four days.

Conclusions

This survey amply bears out the contention, made in the opening paragraphs, that a really critical review of such a mass of diverse observations is not yet possible. Nevertheless it is clear that some interesting lines of investigation have been opened up and that antibiotics may prove to be useful tools for the plant physiologist. The antibiotics include a number of metabolic inhibitors of great specificity and potency. It is therefore not surprising that among their effects on plants some striking inhibitions have been noticed; among the more interesting of these are inhibition of root growth, inhibition of pigment production and plastid formation, and the toxic effects of methionine antagonists on leaves. The stimulation of plant growth by antibiotics, reported by several workers, seems at present quite inexplicable, but it obviously merits further investigation. Finally, attention

XUM

may be drawn to the studies of translocation of antibiotics in plants; such studies are essential to a proper understanding of systemic fungicidal or bactericidal action, but it is likely that they will also increase understanding of the physiology of translocation.

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SOLUBLE OXIDASES AND THEIR FUNCTIONS[1,2]

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Introduction

This review will attempt to evaluate the present status of our knowledge regarding those oxidases whose comparative ease of preparation in a highly purified state has led to the term "soluble oxidase." Such oxidases are in direct contrast to those oxidases, e.g., cytochrome oxidase, which have stubbornly resisted attempts to separate them from cytoplasmic inclusions and to purify them as single entities.

The term "soluble oxidase" is an arbitrary one since, as will be considered in detail below, some oxidases heretofore considered to be soluble have been shown to be associated with cytoplasmic inclusions, albeit the binding may not be strong. The finding that some oxidases are associated, in the cell, with cytoplasmic inclusions is not unexpected and reflects a trend toward the cessation of general interest in highly purified single enzyme entities, a trend accompanied by more general consideration of the function of oxidative enzyme systems in relatively crude cell homogenates and fractions derived therefrom. The problems and challenges of the intracellular localization of enzymes in plant cells have been considered by Weier & Stocking (1), Millerd & Bonner (2), Hackett (85) and excellently summarized by Goddard & Stafford (3).

This review will not attempt a complete literature coverage nor, because of distribution and translation problems, will the Russian literature be considered. Primary emphasis will be placed on critically evaluating the often controversial aspects of the function of the relatively few "soluble oxidases" that are found in plants; most of the material in this review will deal with higher plants. The definition of an oxidase as an enzyme capable of transferring electrons to oxygen will be strictly adhered to; the redundant term "terminal oxidase" will be avoided wherever possible. There are several excellent reviews of general interest relating to the possibility of different oxidases functioning as catalysts in the transfer of electrons to oxygen in plant tissue respiration; of pertinence here are those of Goddard & Meeuse (4), James (5), and Hill & Hartree (6).

[1] The survey of literature pertaining to this review was completed in December, 1956.

[2] The following abbreviations will be used: AA, ascorbic acid; BAL, 2,3-dimercaptopropanol; DHA, dehydroascorbic acid; DPN, diphosphopyridine nucleotide; DPNH, diphosphopyridine nucleotide (reduced form); GSSG, glutathione; GSH, reduced glutathione; IAA, indoleacetic acid; TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide (reduced form).

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Ascorbic Acid Oxidase

Owing to the relatively gradual acceptance of the presence and importance of cytochromes in plant tissues, much effort has been devoted to the study of oxidases other than cytochrome oxidase, which could conceivably play a role in the transfer of electrons to oxygen in cellular respiration. Ascorbic acid oxidase has been often proposed as the oxidase playing the above-mentioned role in the respiration of many plant tissues; such proposals are partly due to the widespread occurrence of the enzyme, in higher plants only, and partly due to the fact that soluble ascorbic acid oxidase preparations are quite insensitive to carbon monoxide, as are many plant tissues themselves.

The purest preparations of ascorbic acid oxidase as well as the most detailed studies of the enzyme's properties have been carried out by Dawson, Nelson, and co-workers. The work of the Columbia University group has been thoroughly reviewed (7, 8) [see also Singer & Kearney (9)]. Unlike the other copper proteins, ascorbic acid oxidase is very substrate specific (7), although, as shown by Johnson & Zilva (10), Dodds (11), and Snow & Zilva (12), the oxidation of various close analogs of ascorbic acid is catalyzed by the enzyme. Because of its widespread occurrence, ascorbic acid itself is generally regarded as the natural substrate for ascorbic acid oxidase; the possibility that other dienols could so function has been ignored.

The highly purified enzyme [Dunn & Dawson (13)] is a deep blue-green in color, has a Q_{O_2} at 25°C. of 1,150,000, an activity equivalent to a turnover number of 246,000 moles of ascorbic acid oxidized per mole of enzyme per minute. At 25°C. and at the partial pressure of oxygen in air, ascorbic acid oxidase is then a powerful catalyst. However, from the data of Thimann, Yocum & Hackett (14) it is doubtful if the turnover number would approach such a large figure at oxygen tensions lower than that in air.

The earlier methods for the preparation of ascorbic acid oxidase have been improved [Dawson & Magee (15)]; the principle improvement being in ease of preparation and in the avoidance of lead acetate as a precipitant. The best preparations of ascorbic acid oxidase that have been described by Dunn & Dawson have a copper content of 0.26 per cent, a molecular weight, by sedimentation, of $146{,}000 \pm 10$ per cent, a value which corresponds to six copper atoms per molecule. The preparation of Dunn & Dawson showed no measurable inhomogeneity when subjected to both electrophoretic and ultracentrifuge analysis. Tadokoro & Tokasugi (16) have described a crystalline protein with ascorbic acid oxidase activity which was prepared from pumpkin, bean sprouts, and cauliflower. Further, the properties of the crystalline protein were those of a nucleotide. Dunn & Dawson (13) have found that their preparations from yellow summer squash, *Curcurbita pepo condensa*, are very unlike those of Tadokoro & Tokasugi. Dunn & Dawson feel that the enzyme prepared from summer squash is a globulin, a conclusion drawn from the solubility behavior of the enzyme. The crystalline preparations de-

scribed by the Japanese workers have not been repeated, and the question of whether there are fundamentally different kinds of ascorbic acid oxidase remains unsettled. Dunn & Dawson (13) have suggested that the crystalline proteins may not have been ascorbic acid oxidase, a view based on the figures for nitrogen, carbon, and phosphorus contents. Tadokoro & Tokasugi did not investigate the copper contents of their preparations.

Since the discovery of ascorbic acid oxidase by Szent-Györgyi in 1931 (17), various investigators have challenged the validity of the enzyme, and it has been suggested that the oxidation of ascorbic acid could be explained in terms of other enzymes, e.g., phenol oxidase or peroxidase, or by ionic copper catalysis. Thus, Barron, Barron & Klemperer (18), McCarthy, Green & King (19), Lampitt & Clayson (20), and Mommaerts (21), have questioned the view that ascorbic acid oxidase is a real entity. Dunn & Dawson have settled the above question permanently in their investigations of the highly purified enzyme, which showed, among other things, the tremendous effectiveness of the enzyme in the catalytic oxidation of ascorbic acid as contrasted to the relative ineffectiveness of ionic copper in this regard. Further, the amount of ionic copper that can be removed from highly purified preparations of ascorbic acid oxidase is small; complete removal of ionic copper by resins did not alter the activity of the enzyme (22). Experiments with Cu^{64} [Joselow & Dawson (23)] have shown that purified enzyme preparations do not exchange the enzyme copper for the radioactive isotope. However, the tracer did exchange with enzyme-copper during aerobic ascorbic acid oxidation. Because of our limitied information concerning the properties of ascorbic acid oxidase, interpretation of the copper exchange experiments is impossible. The investigations cited above reemphasize the importance of copper and point out the firm protein-copper binding.

A curious feature of the purified oxidase is the inactivation that occurs during the oxidation of ascorbic acid. The reaction inactivation of ascorbic acid oxidase has been studied in detail by Dawson and co-workers (22, 24), but there has been no reasonable explanation of this inactivation. Curiously, small amounts of catalase or peroxidase prevent inactivation (24). Hydrogen peroxide is not a primary reaction product (25; see also 7), but in view of the above-mentioned effects of catalase it would seem that this problem should be reconsidered. It is interesting that "cell wall" ascorbic acid oxidase does not exhibit the reaction inactivation (35).

Highly purified solutions of ascorbic acid oxidase are blue-green in color (13), a color which may be observed with partially purified preparations. When a solution of the purified enzyme is placed under anaerobic conditions, or placed under a low oxygen tension in the presence of ascorbic acid, the color changes to a light yellow-brown. A similar behavior was described by Keilin & Mann (26, 27) with partially purified laccase. Keilin & Mann showed that the blue color of laccase was associated with a polysaccharide which adhered closely to the enzyme and was apparently involved in the catalytic activity of laccase. The numerous tests of purity that have been

XUM

applied by the Columbia workers to their purified ascorbic acid oxidase have shown no signs of inhomogeneity. Like laccase, it can be assumed that the blue color, along with its reversible bleaching, is an essential and characteristic feature of the enzyme, a feature that should be exploited in optical investigations of tissues and particulate preparations. In view of the highly colored nature of ascorbic acid oxidase (deep blue-green), it is curious that there are no complete absorption spectra of the enzyme. The Columbia workers have shown that between the region of 200 mμ and 500 mμ there is one sharp protein band with a maximum at 276 mμ.

When one considers the properties of ascorbic acid oxidase as compared to those of phenol oxidase, it is noteworthy that the only two things that are strikingly different between the two enzymes are their color and their behavior toward carbon monoxide. Phenol oxidase when pure is a colorless protein and is easily inhibited by carbon monoxide, while ascorbic acid oxidase is relatively insensitive to carbon monoxide. Even the question of the sensitivity of ascorbic acid oxidase toward carbon monoxide is not clear. James (28) finds the enzyme of barley unaffected while appreciable inhibition of the enzyme in pumpkin has been found (29, 30); in the latter two instances the data are suggestive of insufficient oxygen in gas mixtures. The effect of carbon monoxide on the highly purified enzyme is unknown. The similarity in color between highly purified ascorbic acid oxidase and purified metallo-flavoproteins is striking, and it is tempting to suggest that ascorbic acid oxidase could be an enzyme very similar, if not identical, to phenol oxidase, but unlike phenol oxidase, complexed to a metallo-flavoprotein. Such complexes have been shown to occur between hemo- and flavo-proteins. [See Mahler (31).] An ascorbic acid oxidase flavoprotein would account for the apparent carbon monoxide insensitivity of ascorbic acid oxidase. Assuming a copper flavoprotein, it would account for the greater amount of copper found in ascorbic acid oxidase as compared to phenol oxidase and would equalize the difference between their molecular weights. Finally, it would be possible to explain the reaction inactivation in terms of flavoprotein production of H_2O_2 during aerobic ascorbic acid oxidation. A final judgment of this matter cannot be made at this time because although ascorbic acid oxidase has been studied in considerable detail and in a highly purified form, there are large gaps in our knowledge concerning the fundamental nature of the enzyme itself. It is also quite true that as far as the mechanism of ascorbic acid oxidation by this oxidase is concerned, we know virtually nothing. Although it is generally assumed that the enzyme acts through a reversible valency change of the copper, this hypothesis has never been subjected to experimental proof.

By definition, ascorbic acid oxidase is an enzyme which is easily brought into solution and hence it is referred to as a soluble oxidase. In the past few years, however, reports have appeared concerning the localization of ascorbic acid oxidase in various fractions of cell homogenates. The possibility that ascorbic acid oxidase could be associated with particulate components of the

cell was first raised by Waygood (32) in his studies of wheat respiration. Since Waygood's work, Newcomb (33), and Bryan & Newcomb (34) have shown that in tobacco pith cells ascorbic acid oxidase is associated with the "cell wall" fraction. More recently Honda (35) has confirmed this finding in the roots of barley seedlings, and Hansl (36) has found that 70 to 90 per cent of the ascorbic acid oxidase of the *Avena* coleoptile and its first leaf is found in the "cell wall" fraction. One major difficulty in studying ascorbic acid oxidase and its localization in the cell is the inherent difficulty of distinguishing between ascorbic acid oxidase and cytochrome oxidase. In view of the fact that in the preparation of mitochondrial fractions from ground cell material only about 20 per cent of the total heme is recovered in the mitochondria and the remainder is found in the "cell wall" fraction, it is evident that the mere fact that the "cell wall" fraction oxidizes ascorbic acid is not conclusive proof of the presence of ascorbic acid oxidase. Both Waygood and Honda have presented carefully prepared evidence concerning the peripheral location of ascorbic acid oxidase, and there is no question that the enzyme is found in the fractions sedimented in a low centrifugal field. However, no evidence is presented relating to the structures that appear in the "cell wall" fraction where ratios of whole cells to cell fragments would seem pertinent.

It is difficult to conceive of a role for a powerful oxidase like ascorbic acid oxidase in the cell wall, a structure which is relatively inert metabolically. It is conceivable, of course, that the enzyme is in some manner associated with the activities of the cell wall during cell division and cell elongation, a possibility that will be considered below.

As has been mentioned above, ascorbic acid oxidase often has been suggested as a catalyst for the transport of electrons to molecular oxygen in those tissues whose respiration is insensitive to carbon monoxide (5). The possibility that ascorbic acid oxidase could play a role in cellular oxidation was, of course, first recognized by Szent-Györgyi (17) in 1931. Since that time numerous such suggestions have been made at one time or another. Thus Waygood (32) has recently implicated ascorbic acid oxidase in the respiration of wheat seedlings and James (5, 37, 38) has repeatedly discussed the role of ascorbic acid oxidase in germinating and ageing barley seedlings. Hansl, ignoring the fact that *Avena* coleoptile growth and respiration are quite sensitive to carbon monoxide, has proposed that since a predominant oxidase of the coleoptile is ascorbic acid oxidase and since, at $10^{-3}M$ concentrations, phenylthiocarbamide and 8-hydroxyquinoline are more inhibitory to growth than is azide, ascorbic acid oxidase plays an important role in auxin mediated aerobic growth. It is surprising that in view of the suggestions that have been made concerning the possible roles of ascorbic acid oxidase in tissue respiration that the affinity of this oxidase for oxygen has not been investigated until very recently. Such an investigation, comparing the affinity of ascorbic acid oxidase for oxygen to the similar affinity of the tissue in question, would give an immediate indication of the validity

XUM

of the hypothesis that the copper protein could play a role in cellular respiration by electron transport to molecular oxygen. Thimman, Yocum & Hackett (14) have compared the rates of oxidation of potato tissues, yeast cytochrome oxidase, and purified ascorbic acid oxidase at different oxygen tensions. The data of Thimann, Yocum & Hackett show quite conclusively that the affinity of ascorbic acid oxidase for oxygen is very low; in air the rate of oxygen uptake was roughly half of its rate in oxygen ($P_{O_2}^{50} = 16$ per cent O_2). The oxygen partial pressure curves for freshly cut potato discs and discs washed for four days were very similar ($P_{O_2}^{50} = 0.5$ to 2.1 per cent O_2) to each other and very different from the ascorbic acid oxidase curve. The above experiments are a clear-cut demonstration that ascorbic acid oxidase cannot function as an oxidase mediating potato tissue respiration, a possibility that had been suggested (39). By analogy from the experiments of Thimanm, Yocum & Hackett it is clear that the general thesis of the participation of ascorbic acid oxidase in catalyzing the transport of electrons to molecular oxygen in plant tissue respiration must be abandoned.

During the past few years James (5, 37, 38) has studied in some detail the respiratory characteristics of barley seedlings and has deduced that the nature of their oxidase changes with growth. He has suggested a progressive replacement of cytochrome oxidase by ascorbic acid oxidase. Honda (40) has recently shown that mitochondrial preparations from barley roots of different ages contain an active cytochrome oxidase as well as an active succinic dehydrogenase-cytochrome oxidase system. Similarly, Fritz & Beevers (41) have shown that cytochrome oxidase is present in the roots and shoots of etiolated pea, wheat, and barley seedlings at all stages of development up to 12 days. The demonstration of cytochrome oxidase in barley seedlings does not provide conclusive proof that ascorbic acid oxidase has no role as an oxidase in their respiration, nor does it explain the response of barley seedlings to various inhibitors. As mentioned above, Honda has also shown that the bulk of the ascorbic acid oxidase activity is associated with the "cell wall" fraction. It is quite clear that the major respiratory activities of plants are found in the cytoplasmic inclusions and as yet ascorbic acid oxidase has not been reported in this fraction. Honda (42), in a further study of metal-complexing agents on barley-root respiration has concluded that, in addition to cytochrome oxidase, an auto-oxidizable flavoprotein is functional in the respiration of wheat roots. As will be discussed below, the flavoproteins, like ascorbic acid oxidase, have a very low affinity for oxygen and function much more effectively in intermediate electron or hydrogen transport to acceptors other than oxygen. Hence it seems very unlikely that Honda's conclusion can be generally accepted. It is much more reasonable to assume that the carbon monoxide insensitivity of barley roots can be interpreted as involving an auto-oxidizable cytochrome component other than cytochrome oxidase. Such an interpretation is well founded on the investigations of washed potato discs (14) and has been suggested to account for a similar behavior in the spadix of the skunk cabbage [Bonner & Yocum (43)].

Considerable interest was provoked when two groups, working independently (44, 45), almost simultaneously announced an artificial oxidative pathway which utilized ascorbic acid oxidase to mediate the transfer of electrons to oxygen. The artificial system was based on the finding that plants contained glutathione reductase, a fact which allowed Mapson & Goddard (44) and Conn & Vennesland (45) to establish an oxidative system which contained, in addition to the TPNH-requiring glutathione reductase, dehydroascorbic acid reductase [Crook & Hopkins (46); Crook (47)] and ascorbic acid oxidase. Szent-Györgyi (17) had previously suggested the possibility of glutathione and ascorbic acid oxidase acting as respiratory carriers. The reactions concerned are:

$$a.\ SH_2 + TPN^+ \xrightarrow[\text{dehydrogenase}]{\text{TPN linked}} TPNH + H^+ + S$$

$$b.\ TPNH + H^+ = GSSG \xrightarrow[\text{reductase}]{\text{glutathione}} TPN^+ + 2GSH$$

$$c.\ 2GSH + DHA \xrightarrow[\text{reductase}]{\text{dehydroascorbic}} AA + GSSG$$

$$d.\ AA + \tfrac{1}{2}O_2 \xrightarrow[\text{oxidase}]{\text{ascorbic acid}} DHA + H_2O$$

$$e.\ SH_2 + \tfrac{1}{2}O_2 \longrightarrow S + H_2O$$

In their investigations, Mapson & Goddard used isocitricdehydrogenase to produce TPNH, reaction *a*; Conn & Vennesland used glucose-6-phosphate dehydrogenase. It can be appreciated that reaction *d* can be mediated by any enzyme capable of catalyzing the oxidation of ascorbic acid, e.g., ascorbic acid oxidase, phenol oxidase, cytochrome oxidase, etc.

In a later communication, Mapson & Moustafa (48) analyzed the extent to which the above reactions participate in the respiration of pea seedlings and deduced that about 25 per cent of the total respiration could pass through such a system. In view of the oxygen affinity data mentioned above, it would seem that the above figure is optimistic if ascorbic acid oxidase mediates reaction *d*. Whatever the limitations of the above system, it is interesting that it represents a mechanism for the reoxidation of reduced TPN, a reoxidation for which very little information is available. Young & Conn (49) have studied a system in avocado mitochondria which couples the oxidation of organic acids to the reduction of glutathione; the oxidation of reduced glutathione was regarded to be catalyzed by ascorbic acid oxidase as in reaction *c* and *d* above. The possibility that avocado mitochondria contains ascorbic acid oxidase is interesting, but there is no definite proof of such a possibility in this work.

Mathews (50), in a study of the oxidation of reduced DPN by green pea seedlings has shown that this oxidation is cyanide resistant and requires ascorbic acid but not ascorbic acid oxidase. Beevers (51) has studied a more complex system which, in the oxidation of reduced DPN, requires the presence of a product of ascorbic acid oxidation but a product which is

XUM

not dehydroascorbic acid. Beevers suggests that there is a linkage between reduced DPNH and ascorbic acid oxidase, a reaction which is cyanide resistant but which is very sensitive to BAL and to *p*-chloromecuribenzoate. The fact that the oxidation of reduced DPN by Beever's preparations is insensitive to cyanide abolishes the notion that ascorbic acid oxidase could be involved and it remains quite clear that a solution for problems of cyanide and carbon monoxide resistant respiration will have to be sought elsewhere. As suggested above, such cases are probably similar to the respiratory characteristics of the aroid spadix. The possible relations of ascorbic acid oxidase systems to the oxidation of reduced DPN have been reviewed recently (52).

On the more practical side, Brown (53), Brown & Steinberg (54), and Brown & Hendricks (55) have studied the activity of ascorbic acid oxidase as influenced by various mineral deficiencies. The activity of ascorbic acid oxidase appears to be a good index of the amount of copper available to a variety of crop plants, the oxidase activity being closely related to the supply of copper and, to a lesser extent, to the supply of manganese and molybdenum.

In conclusion, therefore, it is obvious that there remains a good deal of work in relation to the properties, the mechanism of action, and the role of ascorbic acid oxidase in tissue respiration. Much remains to be done in elucidating the structure and the relation of the copper to the protein of the enzyme; a demonstration of a change in valency of the copper during oxidation and reduction would be considered essential and, finally, any possible role of ascorbic acid oxidase in tissue respiration should be considered in the light of (*a*) systems which are very aerobic in nature and hence can use an enzyme of relatively low oxygen affinity, or (*b*) systems in which ascorbic acid oxidase utilizes acceptor substances other than molecular oxygen.

Phenol Oxidase

The name "phenol oxidase" is used to include all terminology describing the enzyme which catalyzes the oxidation of mono- and ortho-diphenolic substances. Hence, tyrosinase, polyphenol oxidase, "DOPA oxidase," potato oxidase, catechol oxidase, etc., are covered in the generic term phenol oxidase. Since its discovery in 1895 (56), phenol oxidase has been the subject of intensive investigation, investigations carried out both on the physical and chemical nature of the enzyme itself and investigations relating to the role of the enzyme in the respiration of higher and lower plants. In spite of much research, the present-day knowledge of the precise nature of the enzyme is limited, and the role of phenol oxidase in cellular respiration remains shrouded in mystery. Because of its ready solubility, phenol oxidase is easily brought into a high state of purity. Thus Kubowitz (57, 58) prepared from potato an enzyme of approximately 50 per cent purity; Keilin & Mann (59), using *Agaricus campestris*, prepared a very pure enzyme, and later, Mallette *et al.* (60), from the same source obtained an equally active preparation. Following the methods used by Keilin & Mann and by the Columbia workers, phenol

oxidase has been prepared from other sources. A fairly pure preparation was made from sweet potatoes by Eiger & Dawson (61), more recently by Yamamoto (131), and from *Lactarius piperatus* [Dalton & Nelson (62)], etc. A detailed outline for the method of purification of the enzyme from *A. campestris* has been recently described by Dawson & Magee (63). Unfortunately, no great innovations have been made in the methods used for the purification of phenol oxidase. The present method of Dawson & Magee suffers from the application of rather drastic procedures such as fractionation with lead subacetate. A more modern approach to the methodology involved in the purification of phenol oxidase is needed by those interested in investigating the fundamental physical properties of this enzyme.

In spite of continued interest in the enzyme throughout a great many years, the present-day knowledge concerning the fundamental properties of phenol oxidase is distressingly small. Although the enzyme that has been reported from various sources [see Dawson & Tarpley (7)] varies somewhat as to color and to copper content there is no question that it is the same enzyme. In the literature to date, there have been only two preparations of a high degree of purity, those of Keilin & Mann (59), and Mallette *et al.* (60). The basic procedures of purification used by both groups of investigators were essentially the same, but there are some differences in the purified enzyme as recorded by these investigators. Thus, the activity (Q_{O_2}) of the preparation of Keilin & Mann as measured in their assay, was 1,170,000 at 20°C., and that of the Mallette *et al.* preparation was 2,640,000 at 25°C. This discrepancy in the activity of the pure enzyme can be ascribed to differences in assay methods. However, there was a considerable difference in the copper content reported for the two different preparations; that of Keilin & Mann contained 0.3 per cent copper while the other contained 0.2 per cent. Further, the preparation of Mallette *et al.* possessed some monophenolase activity while that of Keilin & Mann had none.

Little is known about the physical properties of the enzyme itself aside from the fact that the highly purified protein has a molecular weight of approximately 100,000, a figure which corresponds to a copper content of four atoms of copper per mole. The Michaelis constant for the catalyzed reaction is unknown, as are all other pertinent data relating to the understanding of a purified protein. Unfortunately, much of the effort that has been given to the investigation of the highly purified protein has been devoted to the controversy of monophenol and *o*-diphenol oxidase activity, a controversy which will be reviewed below.

Estimation of the enzyme.—There are at present three basic methods proposed for the estimation of the enzyme. The method used by the Columbia investigators, the chronometric method, is described in detail by Dawson & Magee (63). The advantage ascribed by the Columbia workers to the chronometric method is the circumvention of side reactions in which *o*-quinones are involved; the *o*-quinones are kept in a reduced state by the presence of ascorbic acid and the method is dependent on the analysis of

ascorbic acid. Such a method is, of course, a variation of that proposed by Keilin & Mann (59), and of Kubowitz (57). The method of assay used by Keilin & Mann is dependent on the direct oxidation of catechol in the presence of enough enzyme to give an extremely rapid oxygen uptake. In order that oxygen does not become limiting, the fluid level is kept small and the manometers are oscillated at extremely rapid rates. When used with care, this is perhaps the most direct and meaningful method of activity estimation. Finally, Smith & Stotz (64) have introduced a colorimetric estimation. Such methods have been used previously but not with the precision that is claimed by Smith & Stotz. Their method depends on the reduction of catechol-produced *o*-quinone by leuco-2, 6-dichlorobenzenoneindo-3′-chlorophenol. Under the proper conditions, the rate of color formation is claimed to be linear and directly proportional to the enzyme concentration for short periods of time.

As mentioned above, many of the investigations of phenol oxidase have centered around the question of monophenolase and *o*-diphenolase activity. It is a well-known fact that during phenol oxidase purification the ability to oxidize monophenolic substances is gradually lost. During nearly thirty years of phenol oxidase investigations three main hypotheses have developed to explain the behavior of the enzyme toward mono- and *o*-diphenolic substances. The first of these hypotheses as outlined by Richter (65) envisaged two distinct enzymes: one for the oxidation of monophenols and another for the oxidation of *o*-diphenols. This hypothesis was rejected by Mallette & Dawson (66). Second was the view, first put forward by Onslow & Robinson (67) suggesting that the oxidation of phenols was limited to *o*-diphenols, the monophenol being converted to an *o*-diphenol by hydrogen peroxide resulting from the *o*-diphenol oxidation. However, Dawson & Ludwig (68) showed that hydrogen peroxide was not formed during *o*-diphenol oxidation. The oxidase of sweet potato and the enzyme laccase do not oxidize monophenols and act only on *o*-diphenols, an observation which had provided support for the above hypothesis. Further support came from Kertész (69, 70, 71) who presented evidence in favor of the view that the conversion of monophenol to *o*-diphenol is catalyzed by free and nonprotein-bound metallic ions, e.g., copper, cobalt, nickel, and vanadium. However, Mason *et al.* (72) have studied this reaction with the use of O^{18} and have shown that 3,4-dimethylphenol is enzymatically converted to 4,5-dimethylcatechol and the O^{18} is found in the *o*-hydroxyl group. Such evidence conclusively demonstrates that the conversion of the monophenol to *o*-diphenol is an enzymatic reaction. The enzymatic nature of monophenol oxidation is in line with the views put forward by the Columbia workers, viz., that both monophenol and *o*-diphenol oxidation are carried out by the same enzyme and that the loss of monophenol oxidizing ability during purification is due to fragmentation of the protein during the purification process. In support of this view, the Columbia workers (7) have demonstrated that the ratio of monophenolase to *o*-diphenolase activity can be varied according to the procedure of purifica-

tion used. It is curious that preparations having different ratios of monophenol and *o*-diphenol oxidizing properties have not been prepared by investigators other than those cited.

A very similar view to the one just expressed was put forward by Keilin & Mann (59) who proposed that monophenol oxidation required a heat stable activator. Again, as in the case of the fragmentation hypothesis, there is no real support for this latter proposal. That the status of monophenol and *o*-diphenol catalyzed oxidation has not been entirely clarified is made even more evident by considering the inactivation that follows the course of oxidation. As is well known, the oxidation of *o*-diphenolic substances leads to a rapid and irreversible enzyme inactivation. On the other hand, the oxidation of monophenolic substances by crude or partially purified enzyme preparations does not lead to such an inactivation; on the contrary, a linear rate of oxidation is obtained for some time. It is true that some *o*-diphenolic substances lead to more rapid inactivation than do others, but if the reactions that have been proposed [see Dawson & Tarpley (7)] for the oxidation of monophenolic substances and *o*-diphenolic substances are correct, then it is difficult to reconcile the fact that even though the oxidations proceed in exactly the same manner, the products of the oxidation have very different effects on the enzyme. The fact that catechol rapidly inactivates phenol oxidase has led many investigators to study other *o*-diphenolic substances; such substances of interest here are those which are found within the plant body itself. The investigations of Onslow [summarized in (7, 53, 73, 74, 75)] show that there is a great diversity of phenolic substances in plants which are capable of being oxidized by phenol oxidase.

A detailed review of inhibitors of phenol oxidase has been given by James (28) and those inhibitors specific to phenol oxidase have been discussed by Dawson & Magee (63) and Dawson & Tarpley (7). In general, the inhibitors diethyldithiocarbamate (dieca), phenylthiourea, phenylthiouracil, and their derivatives have been used to distinguish copper proteins from other types of metallo-proteins. Hill & Hartree (6) have discussed the pitfalls that may be encountered in the use of the inhibitors mentioned above. Volkmann & & Beerstecher (76) have introduced recently the use of 3,4-dichlorophenylserine, a substance which they consider to be very specific for copper; it is a powerful inhibitor of both ascorbic acid oxidase and phenol oxidase, the inhibition being relieved by the addition of ionic copper. In addition to specific metal-combining substances, various compounds have been shown to cause a competitive inhibition of phenol oxidase. Thus, Richter (77) suggested the use of various phenolic substances which he considered to act competitively, a suggestion that has been used by Bonner & Wildman (78), Hackney (79) and others. Flesch (136) has shown that melanin formation is inhibited by sulfhydryl reagents, an observation that has been studied by Suzuki (137). The phenol oxidase of *Scopolia japonica* was shown to be inhibited by thiosulfate and thioglycolate, although the concentrations required are relatively high. The thiosulfate inhibition is competitive (137).

Finally, many of the investigations concerning the role of phenol oxidase in plant respiration have utilized general metal combining agents such as cyanide, azide, sulfide, and carbon monoxide. Special emphasis has been placed on the fact that the carbon monoxide-phenol oxidase complex does not dissociate in light [Keilin (80)]. The absence of light sensitivity in carbon monoxide inhibited respiration is not conclusive evidence that an enzyme other than cytochrome oxidase is operative. Keilin (81) has shown that there are cytochrome oxidases which behave in a similar manner to that of phenol oxidase. Thus, Keilin (81) showed the carbon monoxide inhibited respiration of *E. coli* is not light sensitive, an observation confirmed by Yamagutchi (134) and extended by him to other bacteria, each of which have well defined cytochromes. Carbon monoxide heme itself is insensitive to light [Keilin (81); Keilin & Hartree (135)].

The classical investigations of Kubowitz (57, 58) on the carbon monoxide inhibition of partially purified potato phenol oxidase have not been reinvestigated. From his work, and by analogy from the behavior of hemocyanin and carbon monoxide, Kubowitz deduced that phenol oxidase copper occurs in the cupric state, is reduced to the cuprous state during the oxidation of substrate, and that carbon monoxide acts on the enzyme only when it is in the cuprous state. It is curious that these deductions have been accepted widely for 19 years and have not been reinvestigated. Further, as in the case of ascorbic acid oxidase, there has been no direct demonstration that the copper of phenol oxidase undergoes a valency change during the oxidation of phenolic substances. The investigations of Mason (72, 82, 83) and of Bonner (84) have reemphasized the importance of electron transport during phenol oxidation, but direct demonstration of a change in protein-copper valency during oxidation is urgently needed. In spite of the plethora of papers on the subject of phenol oxidase, we are at present faced with the melancholy conclusion that while being wonderfully well informed, we still remain woefully ignorant concerning the fundamental properties of the enzyme.

Phenol oxidase has been regarded as a soluble enzyme because it is an enzyme that is readily brought into solution. In the past few years, however, we have been presented with increasing evidence that phenol oxidase occurs in the cell in association with cytoplasmic inclusions. It may well be that the ready solubility of the enzyme has been misleading and that the role of phenol oxidase can be more critically appraised from investigations of particulate preparations. Li & Bonner (86) presented evidence showing that the phenol oxidase of tea leaves was associated with the grana, a proposal that was supported by the work of Arnon (87) who observed that in beet leaves phenol oxidase was definitely associated with chloroplasts. The presence of phenol oxidase in mitochondrial fractions has been subsequently suggested by McClendon (88), who, in a study of the intracellular localization of enzymes in tobacco leaves, found phenol oxidase present in all of the

XUM

fractions that he tested. The enzyme was easily washed off the particulate fractions, a fact that may be explained by the homogenizing technique used by McClendon. More recently, Bonner (84) has shown that phenol oxidase is present in mitochondrial fractions obtained from mushroom and from etiolated mung bean seedlings. In the latter instance, the enzyme appeared firmly bound. Similarly, Haskins (133) has reported phenol oxidase to be associated with particulates sedimented at 40,000 g. It may be that in plants containing phenol oxidase, the ratio of soluble to bound phenol oxidase can serve as an index of the integrity of isolated cytoplasmic inclusions.

The fact that phenol oxidase is bound to cytoplasmic inclusions could explain the results of Keilin & Mann (59) showing the yield of soluble enzyme to be greatly increased by allowing aqueous mushroom extracts to stand some hours. Presumably, the increased yield of enzyme resulted from the disruption of cytoplasmic particles and the consequent release of the enzyme into solution.

Phenol oxidase is both a very powerful catalyst of phenol oxidation and a catalyst capable of acting on a variety of phenolic substances. Because of these considerations and because of the widespread distribution of phenol oxidase in the plant world, numerous attempts have been made to implicate the enzyme as a catalyst mediating the final reaction with oxygen in cellular respiration. Such attempts, initiated over 20 years ago by Onslow (73) and Boswell & Whiting (89) have been elaborated and extended by numerous investigators. The basic supposition of all proposals involving phenol oxidase in plant respiration has been centered on the reduction of the *o*-quinone formed from *o*-diphenol oxidation, reduced coenzymes, ascorbic acid, or "quinone reductase" (90, 132) being suitable reducing agents. The *o*-diphenol complex would then act as a couple, transporting electrons from reduced coenzyme to copper and finally to oxygen. Such a mechanism was first used by Kubowitz (57, 58), who utilized TPNH as a reducing agent, and since those classical investigations numerous people have employed similar systems. Using such systems, it has been possible to set up artificial oxidation systems which are capable of oxidizing various organic acids in the presence of the suitable dehydrogenase, coenzyme, catechol, and phenol oxidase. Numerous investigators have proposed that the main portion, if not all, of the respiration of a variety of plant tissues proceeds through a system similar to that suggested above. Thus, Sreerangachar (91) has proposed that phenol oxidase acts as the oxidase in the respiration of tea leaves and stems. His work has received support from others [see Nelson (75)]. More recently, Bonner & Wildman (78) have come to similar conclusions concerning the respiration of spinach leaves. Their study was based on the inhibition produced by *p*-nitrophenol, a substance which was regarded as being a selective, competitive inhibitor of phenol oxidase. Stenlid (92), although agreeing in principle with Bonner & Wildman, has not agreed with the process by which they arrived at their conclusion, and it was not until a more recent work of

Simon & Beevers (93), which showed that the respiration of yeast is strongly inhibited by *p*-nitrophenol, that the specificity of this inhibitor was seriously questioned.

Recently, Markh & Fel'dman (138) have suggested that 50 per cent of the oxygen uptake of tomato fruit is mediated by phenol oxidase. Almost all of the investigations purporting to show the phenol oxidase mediation of electron transport to oxygen in tissue respiration have failed to take into consideration two important points: (*a*) the properties of and methods for the investigation of the cytochrome components of plant tissues, and (*b*) the affinity of phenol oxidase for oxygen. At the present time, most plant physiologists are aware of the importance of cytochrome mediated electron transfer and the above questions are not often overlooked today. However, it is curious that the affinity of phenol oxidase for oxygen remains almost completely uninvestigated. Apart from some tentative results of Kubowitz indicating a low affinity of the enzyme for oxygen, there have been no other investigations of this important matter. If the affinity of phenol oxidase for oxygen is low, as indicated by Kubowitz, then investigators should search for an alternative role for the enzyme, a role involved in electron transport to acceptors other than oxygen. That phenol oxidase can be involved in such an electron transport has recently been shown by Bonner (84) who has demonstrated that cytochrome-*c* is a very efficient electron acceptor for *p*-cresol oxidation.

From the investigations of Bonner, investigations reinforced by the hypothesis of Mason (83) that electron transport is obligatory for phenol oxidation, it would appear that the role of phenol oxidation in plant respiration should be evaluated in terms of electron transport in anaerobic systems. It is possible that phenol oxidase could function in electron transport to cytochrome-*c* in mitochondria and in electron transport to cytochrome-*f* in the chloroplasts. In summary, it is obvious that the properties of the purified enzyme as well as the relatively crude mitochondrial-bound enzyme should be thoroughly investigated. There is need for a much improved method for the preparation of phenol oxidase so that fundamental work dealing with the properties of the enzyme can be more readily carried out.

Laccase

Laccase is an enzyme which has been very much neglected in recent years. Apart from the preparations of Keilin & Mann (26, 27) and of Gregg & Miller (94), very little work has been carried out in attempts to purify the enzyme and study its properties. Keilin & Mann (26) have prepared laccase from the latex of the Indo-Chinese lacquer tree. The copper content of their enzyme was 0.15 per cent but they found a considerable amount of polysaccharide present in the preparation and, correcting for the polysaccharide, the copper content was .34 per cent, a figure in closer agreement with the two previously discussed copper proteins. A higher degree of purification was achieved later (27). A point that has been discussed frequently has

been the kind of metal associated with this enzyme, a problem which has been resolved by Tissières (95) who, with a partially purified laccase preparation, removed the copper by dialysis against cyanide and found that the enzyme could only be reactivated by the addition of copper. Like ascorbic acid oxidase, the laccase preparations of Keilin & Mann are blue-green in color, the color disappearing reversibly on the addition of substrates. Keilin & Mann have associated the color with the presence of polysaccharides but this observation has not been confirmed elsewhere. The color as related to the activity has already been discussed. Like phenol oxidase, the relatively impure preparations of laccase will oxidize an assortment of substrates; there is no activity on monophenolic substances, but laccase catalyzes the oxidation of *p*-phenylenediamine, hydroquinone, and *o*-diphenols. Keilin & Mann have shown that ascorbic acid is directly oxidized by laccase, but the ability to oxidize ascorbic acid diminishes with increasing purity of the enzyme. Bertrand (96, 97), on the other hand, has suggested that highly purified laccase oxidizes ascorbic acid directly, and in addition, has shown a laccase-catalyzed oxidation of reduced cytochrome-*c* (98, 99). The possibility that laccase can utilize cytochrome-*c* as an electron acceptor, the reverse of Bertrand's reaction, would be in agreement with the phenol oxidase-cytochrome-*c* reaction discussed above. The work of Bertrand has not been repeated; the catalytic nature of laccase on cytochrome-*c* oxidation needs confirmation.

Like the other copper proteins, laccase in inhibited by cyanide, azide, dithiocarbamate, etc., but unlike phenol oxidase and like ascorbic acid oxidase, it is not appreciably inhibited by carbon monoxide (26). It is difficult to distinguish between laccase and cytochrome oxidase by means of ascorbic acid or *p*-phenylenediamine oxidation and between phenol oxidase and laccase by means of polyphenol oxidation; therefore considerable care must be used to distinguish critically between the copper proteins and cytochrome oxidase.

Lindeberg (100 to 104) has studied the laccase-phenol oxidase interrelationships in various *Hymenomycetes*, organisms that probably contain a cytochrome system but in addition, produce large quantities of copper-proteins. The close relationship between phenol oxidase and laccase is emphasized by Lindeberg's work which shows that in general, phenol oxidase is found in the mycelia of the fungi that he investigated and laccase tends to be present in the fruiting bodies. Curiously, Lindeberg found a tendency for laccase to be excreted into the medium whereas phenol oxidase was generally not so excreted. Further interrelations between laccase and phenol oxidase have been pointed out in the common mushroom *Psalliota bispora albida* (104) where he found, in contrast to the above, that the mycelia contained laccase and the fruiting bodies phenol oxidase. Separate extractions of the rhizomorphs showed the presence of both enzymes. It can be concluded, therefore, on the basis of the work of Lindeberg *et al.*, that there is an extremely close relationship between phenol oxidase and laccase. It may be

XUM

that there is a free interconversion of one enzyme to another, a fact which suggests the identical nature of the two enzymes. If the two enzymes are identical, the differences in substrate specificity could be modified by the presence or absence of cofactors, a possibility previously suggested by Keilin & Mann (59) in their consideration of the monophenolase and *o*-diphenolase activities of phenol oxidase. As discussed previously, the insensitivity of laccase and ascorbic acid oxidase to carbon monoxide must, in some manner, be associated with the blue pigment.

The role of laccase in the respiratory behavior of plant tissues remains completely unknown. It is interesting that Higuchi, Kawamura & Morimoto (105) have investigated some of the properties of laccase prepared from the latex of the Japanese lac tree and from the wood rotting fungus, *Coriolus versicolor;* preparations from both these sources catalyze the oxidation of coniferyl alcohol. The oxidation of coniferyl alcohol is inhibited by sodium azide (2×10^{-3} *M*), by salicylaldoxime (3.3×10^{-3} *M*) and is not inhibited by carbon monoxide. Potato phenol oxidase preparation did not catalyze coniferyl alcohol oxidation, and these workers have suggested that laccase functions in lignin formation, a suggestion which has been previously made in the case of phenol oxidase by Mason (83). In view of the conflicting reports concerning laccase and its function in plant respiration, it may be concluded that no real progress in this field will be made until there are more serious studies of the nature and properties of this enzyme. The behavior of laccase toward carbon monoxide and the characteristic color of laccase suggests a very close relationship to ascorbic acid oxidase; on the other hand, the substrates utilized by laccase suggests a very close relationship to phenol oxidase, and, on the basis of such relationships, it is suggested here that the three enzymes are extremely closely related to each other, if not identical. Justification or rejection of such a proposal can only await the test of time, but basic information is imperatively needed concerning the physical and chemical properties of all three of the above mentioned copper proteins. Finally, by analogy to the ascorbic acid oxidase and phenol oxidase, one can conclude that undoubtedly the oxygen affinity of laccase is relatively low and a role for laccase in plant respiration should be sought, not as an enzyme catalyzing the transfer of electrons to oxygen, but as one which utilizes some other acceptor in intracellular electron transfer.

Flavoproteins

The flavoproteins have enjoyed the distinction of being regarded as capable of participating in anaerobic electron transport as well as direct transport to oxygen, the latter reaction resulting in the production of hydrogen peroxide. Because of their ability to react directly with oxygen, flavoproteins have frequently been evoked to explain the respiratory behavior of various plant tissues whose response to carbon monoxide, cyanide, or azide has not fitted in with the classical interpretation of the effectiveness of these poisons on various oxidative enzymes. The above deduction has been

made in spite of the fact that it had been known for many years that flavoproteins react much more efficiently with the hemoproteins than with oxygen. Thus, Haas *et al.* (106) showed that at low oxygen tensions cytochrome-*c* reductase reacts 10^6 times faster with cytochrome-*c* than with molecular oxygen. From the investigations of Theorell (107), Haas *et al.* (106), Keilin & Hartree (108), Horecker & Heppel (109), Horecker (110), and Laser (111), pertaining to the oxygen affinity of flavoproteins and the affinity of characterized flavoproteins for substances other than oxygen, it is quite clear that the efficiency of flavoproteins in respiratory processes is associated with anaerobic hydrogen and electron transfer, not with transfer to oxygen. At the present time the suggestion that flavoprotein acts in plant tissue respiration by direct transfer of electrons to oxygen has given way to participation of cytochromes, for example, in the studies of the spadix of skunk cabbage by Bonner & Yocum (43) and Yocum & Hackett (112), and it is the feeling of this reviewer that flavoproteins should no longer be regarded as "terminal oxidases." It may well be that in some isolated instances, which will be cited below, flavoproteins do play a role in transferring electrons to oxygen, but such a role would be a minor part of the overall tissue respiration and would of necessity be associated only with special, highly aerobic oxidative pathways.

Amine Oxidase

In an extension of earlier work (113), Mann (114) has achieved considerable purification of amine oxidase from pea seedlings. Investigation of the purified enzyme has led Mann to conclude that previously described mono- and di-amine oxidases [Cromwell (115); Werle and co-workers (116 to 119); Zeller (120)] are all one enzyme—plant amine oxidase. The purified enzyme is inhibited by cyanide, semicarbazide, diethyldithiocarbamate, etc., facts suggesting a metallo-protein although no absorption spectra data are given. Mann did find copper and manganese to be present in very small amounts, but the possibility of a metallo-protein was not pursued further. Hydrogen peroxide is a primary reaction product of the oxidation of amines and was shown to produce secondary oxidations as well as to inactivate the enzyme itself. Catalase protects the enzyme from inactivation as do thiourea and phenylthiourea, the latter competing with the enzyme for H_2O_2. The product of amine oxidation is generally regarded as the corresponding aldehyde [Zeller (120)]. Oxidation products were not investigated by Mann. The importance and role of amine oxidase in tissue respiration is little understood.

α-Hydroxyacid Oxidase

As discussed in a previous review (6), Kenten & Mann (121) have demonstrated the peroxidatic oxidation of bivalent manganese in the presence of hydrogen peroxide or hydrogen peroxide-producing systems, peroxidase, and a substrate (H-donor). These investigations have been con-

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tinued and Kenten & Mann (122) have described a Mn^{++} peroxidase system that oxidizes various dicarboxylic acids. The acids studied were oxalate, oxaloacetate, ketomalonate, dihydroxytartrate, and dihydroxymaleate. Such oxidations are dependent on concomitant reactions of an oxidative nature i.e., oxidation of reduced flavoproteins by molecular oxygen which produces H_2O_2, and on the presence of peroxidase. Hence, the role and possible importance of such oxidations in plant tissues remains obscure.

Prior to the above investigations, Clagett *et al.* (123) reported the presence of a soluble enzyme in the green parts of higher plants which catalyzed the oxidation of glycolic acid to formic acid and of lactic acid to pyruvic acid. The enzyme, glycolic acid oxidase or α-hydroxyacid oxidase, has been studied in more detail by Kenten & Mann (124) and by Noll & Burris (125). Kenten & Mann found that the hydrogen peroxide-producing system, flavoprotein, which they had used in their manganese oxidation experiments (121) could be replaced by α-hydroxyacid oxidase. Investigation of the purified enzyme showed that H_2O_2 was a product of lactate, glycolate, and glyoxylate oxidations, a fact not established previously (126).

The preparation and properties of highly purified glycolic acid oxidase were described by Zelitch & Ochoa (127). It was shown that the enzyme contained riboflavin monophosphate as its prosthetic group, that only one enzyme was involved in the oxidation of either lactic or glycolic acid, and, in agreement with Kenten & Mann, that H_2O_2 was a reaction product. Noll & Burris (125) demonstrated the presence of glycolic acid oxidase in 16 plant families. The enzyme was found in detectable amounts in etiolated tissues but illumination greatly enhanced its activity. Glycolic acid oxidase was found to be inhibited by sulfhydryl combining reagents. At the present time, the role of this oxidase in plant tissues is not understood but has been discussed in terms of possible functions in photosynthesis (128, 129) and, more recently, in the transfer of electrons to oxygen (130). It is difficult to assess the latter interpretation in view of the lack of information concerning the activity of the enzyme in tissues. It is probable, however, that flavoproteins play an insignificant role in the direct transfer of electrons to oxygen.

Lipoxidase

Lipoxidase is a soluble enzyme of widespread occurrence in the plant kingdom, one that has been prepared in a crystalline form and thoroughly studied by many investigators, and yet we know relatively little concerning the role of this enzyme in plant tissue respiration. The properties of lipoxidase as well as the literature are summarized by Holman & Bergström (139). Lipoxidase has received considerable attention from food investigation laboratories because of the coupled peroxidatic oxidation of carotene which accompanies the oxidation of unsaturated fatty acids, a fact that was first pointed out by Sumner & Sumner (140). The occurrence and properties of lipoxidase in a variety of plants have been studied by Tappel and collaborators (141 to 144). The suggestion of Walsh & Hauge (145) that the lipoxidase

of alfalfa differed from the conventional soy bean enzyme was shown to be untenable, and in fact, the enzymes from the two different sources proved to be identical. A curious and characteristic feature of lipoxidase is the fact that neither heavy metal enzyme inhibitors, flouride, nor arsenicals, are effective inhibitors. The only efficient inhibitors of lipoxidase appear to be antioxidants such as nordihydroguaretic acid, propyl gallate, hydroquinone, α-naphthol, etc. (139, 144). The effects of catalase were not investigated by the above workers.

The rapid oxygen uptake exhibited by freshly prepared corn seedling homogenates has been shown by Fritz & Beevers (146) to be caused by lipoxidase activity. Fritz & Beevers (147) also have investigated the "dye oxidase" of Smith & Stotz (64). The term "dye oxidase" has been applied to the oxidation of reduced 2,3′,6-trichloroindophenol, a reaction found to be catalyzed by plant homogenates from various sources. The dye oxidation was shown by Fritz & Beevers to be catalyzed by lipoxidase and its substrates; neither substrate nor enzyme was effective alone. A curious feature of the dye oxidation was that the dye was bleached (reduced) after short periods of time and the bleaching was irreversible. The dye oxidation was shown to be nonperoxidatic in nature by the insensitivity of this system to cyanide and azide and by the absence of catalase inhibition. In contrast to the lipoxidase of soy bean, potato lipoxidase caused a rapid irreversible bleaching of the dye but the oxidation was inhibited by cyanide. In the face of very little evidence, Fritz & Beevers have made the tenuous suggestion that lipoxidase could play a role in the oxidation by molecular oxygen of reduced cysteine, reduced glutathione, reduced TPN, and reduced DPN, lipoxidase being considered as the catalyst involved in the transfer of electrons to oxygen. The oxygen affinity of lipoxidase remains unknown, but in view of the fact that the oxidation of fatty acids involves a formation of organic peroxides (139) and, further, since Fritz & Beevers showed that in the intact corn seedlings negligible lipoxidase activity could be observed, it can be concluded that the activities of this enzyme are highly aerobic. The suggestions of Fritz & Beevers pertaining to a role of lipoxidase in electron transport to oxygen are untenable.

Another soluble enzyme, described by Stumpf and co-workers (148), has been obtained from peanuts. This carries out a peroxidatic oxidation of fatty acids. In the first investigation, it was shown that the enzyme required activation by the addition of L-α-hydroxy aliphatic acids before fatty acid oxidation could be obtained. This requirement has been shown to be a "priming requirement" (149), the α-hydroxy acid being oxidized by glycolic oxidase, present as an impurity in the preparation. The resulting peroxide then acts in the fatty acid oxidase system. In contrast to lipoxidase, the enzyme described by Stumpf *et al.* is sensitive to azide and cyanide but is not inhibited by catalase. The mechanism of this reaction remains unknown.

It is interesting that many of the above named enzymes, or enzyme systems, appear to be flavoproteins, either produce peroxides or require

peroxides for the consummation of the oxidation, and require molecular oxygen. These facts, taken together, suggest an important role for peroxide in tissue respiration. It is noteworthy that there are no recorded observations of hydrogen peroxide-peroxidase complexes in cells or tissues other than that found by Chance in *Micrococcus lysodeikticus* (150). The conclusion of this reviewer is that there is no evidence that any of the so-called "soluble oxidases" perform as oxidases in plant tissues.

"Indoleacetic Acid Oxidase"

The term "indoleacetic acid oxidase" has been used to describe the capacity of various plant preparations to catalyze the oxidative destruction of indoleacetic acid (IAA) (151 to 156). The properties of IAA oxidase were described by Galston *et al.* (155) who proposed that the system consisted of a flavoprotein and peroxidase. The peroxidation of IAA was regarded as resulting from the action of peroxidase and H_2O_2, the latter being produced by direct oxidation of reduced flavoprotein. It was further suggested that the substrate of the flavoprotein could be IAA itself. No data in support of the stoichiometry of such a coupled oxidation were presented. Galston *et al.* (155) further demonstrated that "pure" (crystalline) peroxidase itself catalyzed the oxidation of IAA, but only in the absence of H_2O_2, a finding that is difficult to interpret.

The peroxidation of IAA has been reinvestigated by Kenten (157). Working with preparations from wax pod bean sap, Kenten found that the IAA oxidase activity could be separated into thermostable and thermolabile portions; the latter could be replaced by peroxidase. Kenten was unable to find evidence of a flavoprotein in either of the two fractions. In agreement with Galston *et al.* (155), Kenten found that peroxidase itself (90 per cent pure) catalyzed the oxidation of IAA; the rate of oxidation was small (*ca.* 80 μl.O_2/60 min.). The rate of oxidation was increased considerably by the addition of an ultrafiltrate of bean sap (*ca.* 260 μl.O_2/60 min.). The ultrafiltrate was thermostable and evidence was presented indicating that its activity was dependent on the presence of peroxidase substrates. The rate of oxidation was further increased by the addition of monophenols and other substances identical or similar to ones previously found to enhance IAA oxidase activity (158, 159). All the activating substances were peroxidase substrates. In contrast to the observations of Galston *et al.* (155), manganese also promoted the oxidation. Curiously, low concentrations of maleic hydrazide considerably increased the rate of IAA oxidation.

Kenten found that the oxidation of IAA was inhibited by low concentrations of catechol, hydroquinone, and *p*-phenylenediamine. The inhibition was interpreted as due to competition of substrate for peroxidase.

Ray & Thimann (160) and Ray (161) have studied a similar IAA oxidizing system, produced by *Omphalia flavida*, the properties and stoichiometry of which are exactly like those previously described, with the exception that the system produced by *Omphalia* has a very acid pH optimum. Ray (162)

has questioned the competitive nature of the inhibition produced by polyphenols. From a study of the polyphenol inhibition of IAA oxidation and of the IAA inhibition of polyphenol peroxidation, Ray has deduced that IAA, in the presence of H_2O_2, reduces peroxidase to Fe^{++}-peroxidase.

The enzyme system produced by *Omphalia* was found to be specific for IAA and indoleisobutyric acids (160). Kenten (163) demonstrated that peroxidase oxidizes IAA, indolepyruvic, and indolebutyric acids, the latter two reactions requiring Mn^{++} and producing H_2O_2. The oxidation of indolepropionic and indolebutyric acids by IAA oxidase of wax pod bean root extracts had previously been demonstrated by Wagenknecht & Burris (152).

A detailed study of the products of IAA peroxidation (160, 161) has shown that the reaction takes place in two spectrophotometrically distinct steps; the first step being the formation of an intermediate which then produces a variety of final products. The properties of the intermediate suggest an aldehyde, but complete identification has not been successful.

There are indications that IAA formation as well as destruction are related to phenol oxidase. Thus, Paleg & Gordon (164) have deduced that in mung beans there is a phenol oxidase-mediated conversion of tryptophan to IAA, an oxidation dependent on the presence of *o*-diphenols. In contrast, Briggs & Ray (165) have demonstrated the inactivation of IAA by mushroom and *Osmunda* phenol oxidase. In this latter instance, it is well worth recalling that both phenol oxidase and peroxidase catalyze the oxidation, in the presence of molecular oxygen, of the same general types of compounds. As has been previously pointed out (6), it is noteworthy that, in general, plants contain one of the copper proteins or peroxidase, rarely both.

It is apparent from consideration of the evidence relating to IAA oxidation that there is no one specific IAA oxidase; rather, we are presented with a variety of IAA oxidizing systems that have been produced as a result of cell rupture. It is difficult to conceive of a role that such highly active and destructive enzyme systems can play in the tissues of plants. If such rapid oxidations are normally carried out by plant tissues, the original discovery of auxin, by the slow process of diffusion, (166, 167) was indeed an impressive accomplishment! The significance of such powerful oxidative systems must be assessed in terms of growth and development as well as the respiratory activities of intact plant tissues.

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XUM

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XUM

AUTHOR INDEX

A

Aach, H. G., 313, 316, 320
Abegg, F. A., 402, 406
Abelson, P. H., 66, 74, 96, 102, 104, 105, 340
Abendschein, P. A., 73, 74
Åberg, B., 153-80; 156, 161, 163, 164, 165, 170, 171, 172, 174, 175, 176
Abraham, A., 380
Abraham, R. G., 44
Abrahamse, S., 223
Abrahamson, E. W., 117, 118
Abrams, G. J. von, 196, 400
Abramsky, T., 58
Adams, D., 34
Addoms, R. M., 47
Adelman, A. H., 124
Adolph, E. F., 2
Agthe, C., 360, 361
Aikawa, I., 146
Akamine, E. K., 295
Alamercery, J., 418, 419
Albaum, H. G., 157
Alcorn, S. M., 418
Aldous, J. G., 341
Alexeeiv, A. I., 263
Alfthan, M., 65, 72, 74, 92
Allard, H. A., 292, 396
Allaway, W. H., 31
Allen, F. L., 138, 147, 148, 149
Allen, M. B., 46, 52, 53, 54, 142, 310, 324, 326
Allen, O. N., 415
Allison, R. K., 138
Allsopp, A., 79
Almestrand, A., 169
Alvim, P. T., 258
Ames, S. R., 434, 435
Amiot, A. F., see Faivre-Amiot, A.
Amlong, H. U., 161, 162, 171, 172, 173
Ammann, A., 415
An, T. T., 86
Anderson, A. J., 52, 95
Anderson, D. B., 139, 250
Anderson, E. G., 395, 401, 402, 406, 408
Anderson, H. W., 415, 419, 421
Anderson, I., 50
Anderson, W. P., 204, 207
Andreae, S. R., 446
Andreae, W. A., 157, 162, 163, 446
Angel, C. R., 189, 207
Angell, H. R., 401
Applewhite, T. H., 335
Arakatsu, Y., 50
Arceneaux, G., 301
Argles, G. K., 217, 223
Arhimo, A. A., 74
Arikado, H., 288, 299
Arisz, W. H., 11, 13, 337, 341, 349, 351, 356, 365, 371
Ark, P. A., 418
Armbrust, K., 86
Arndt, C. H., 242
Arnold, W., 121, 127, 128
Arnon, D. I., 31, 43, 44, 46, 52, 53, 54, 58, 106, 137, 139, 142, 147, 148, 310, 324, 438
Arnow, P., 310, 321
Arny, D. C., 415
Aronoff, S., 138, 142, 143, 146, 310, 362, 363, 364
Arreguin, B., 145, 407
Arthington, W., 90
Arvidsson, I., 257
Asboe-Hansen, G., 78
Ashby, E., 271
Ashby, W. C., 159, 165
Ashcröft, R. T., 49, 56
Ashida, J., 50
Ashton, F. M., 275-308; 286, 287, 288, 291, 292, 299
Ashworth, P. R., 47, 57, 381
Askenasy, E., 237
Aso, K., 189, 191, 192, 197, 198, 202, 207
Athenstädt, H., 227
Atherton, P. G., 299
Atkins, R. E., 402
Atwood, S. S., 401
Audia, W. V., 194, 203, 206, 207, 208, 210
Audus, L. J., 155, 157, 158, 159, 161, 164, 171, 172, 175
Auerback, B. C., 44
Avery, G. S., 160
Ayräpää, T., 340, 341
Ayres, A., 284, 285, 286

B

Bagenal, N. B., 218, 220
Bailey, I. W., 243, 253
Baker, F. E., 34
Baker, H., 237
Baker, J. E., 51
Baker, R. S., 446
Bakke, A. L., 244
Balch, R. T., 280, 289, 301, 302
Baldacci, E., 182, 184, 185, 186, 201, 207
Ball, N. G., 168
Balston, J. N., 67
Bandurski, R. S., 398, 402
Banfield, W. M., 253
Bangdiwala, I. S., 283, 299
Bannister, T. T., 120, 126, 129
Baret, R., 86
Barger, G., 86
Barham, W. S., 404
Barnes, A. C., 295
Barnes, D. K., 239
Barret, F. A., 258
Barron, A. G., 429
Barron, E. S. G., 148, 429
Barrows, H. L., 48
Bartel, A. T., 267
Bartels, P., 342
Barton, A. D., 73
Barton, L. V., 196, 207, 414, 415, 420
Barton, N. L., 37
Barton-Wright, E. C., 408
Bartsch, A. F., 315
Bartz, Q. R., 72
Bassham, J. A., 74, 89, 310, 444
Batchelor, L. D., 229
Bathurst, N. O., 97
Bauer, L., 350, 351, 354, 355, 356, 357, 362, 364, 370
Baver, L. D., 289, 298
Bawden, F. C., 103
Beadle, G. W., 393, 403
Beadle, N. C. W., 376, 382
Beakbane, A. B., 217-36; 218, 220, 221, 222, 226, 228, 230, 231
Beams, H. W., 142
Beauchamp, C. E., 301
Beck, E. W., 404
Becker, R. S., 119, 120
Beckman, C. H., 243, 253
Beerstecher, E., Jr., 437
Beevers, H., 341, 342, 432, 433, 440, 445
Bein, M., 169, 415, 418, 419
Beingessner, H. F., 223
Bell, E. A., 86
Bell, W., 415
Bella, D. D., see Della Bella, D.
Belval, H., 441
Benedict, H. M., 250
Ben-Ishai, R., 100
Benitez, A., 116
Bennet-Clark, T. A., 29, 155, 168, 175
Bennett, C. W., 358, 359, 360

XUM

Bennett, E. L., 383
Bennett, M., 228
Bennett, M. C., 337, 341
Benoiton, L., 73, 83, 92
Benson, A. A., 39, 74, 89, 310, 444
Bentley, J. A., 155, 156, 158, 159, 165
Berbee, J. G., 243, 253
Berg, A. M., 72, 82, 92
Berger, E., 258
Bergquist, A., 74
Bergström, S., 444, 445
Bernfeld, P., 20
Bertrand, D., 441
Bertrand, S., 434
Besson, G., 415, 418, 419
Bexon, D., 29
Bickle, A. S., 158, 159, 165
Biddulph, O., 31, 39, 46, 59, 359, 361, 362, 363
Biddulph, S. F., 38, 39, 40, 46
Bidwell, R. S. G., 84, 101, 102, 104, 105
Biebl, R., 337
Bierhuizen, J. F., 43, 53
Billings, W. D., 375-92; 377, 379, 380, 382, 387, 388
Binkley, W. W., 278, 280, 281
Bird, R. B., 240
Bishop, N. R., 141, 144, 145, 146
Björkman, L., 310, 318
Björkman, M., 310, 318
Blaauw, O. H., 141, 143, 144, 146
Black, J. N., 377
Black, M., 378
Black, S., 70, 74, 92
Blackburn, F. H. B., 283
Blackman, G. E., 377, 384, 385
Blair, D., 221
Blair, D S., 221, 223
Blair, M. G., 278, 280
Blaney, H. F., 298
Bledsoe, R. W., 38, 39
Bleidner, W. E., 85
Blinks, A. H., 4
Blinks, L. R., 4, 8, 309, 322
Bliss, L. C., 377, 388
Block, R. J., 67, 69
Bocchi, A., 202, 207, 208
Bode, H. R., 237, 242, 246
Bogen, H. J., 272, 335, 336
Boggess, W. R., 257
Bogorad, L., 417
Böhning, R. H., 363
Bohonos, N., 310, 321
Bokura, U., 181
Bolas, B. D., 229
Bold, H. C., 138
Bolhuis, G. G., 39, 48
Bolin, I., 74
Boll, W. G., 55
Bollard, E. G., 68, 79, 85, 87, 89, 230
Bolle-Jones, E. W., 47
Bolli, M., 266
Bolton, E. T., 66, 96, 102, 104, 105, 340
Bonazzi, A., 285, 291
Bonner, D. M., 74, 90, 205
Bonner, J., 42, 74, 105, 145, 157, 162, 163, 164, 168, 169, 335, 336, 383, 407, 419, 427, 437, 438, 439, 446
Bonner, W. D., Jr., 427-52; 432, 438, 439, 440, 443
Borck, B., 221
Borden, R. J., 281, 286, 298, 299, 300
Borgström, G., 165
Bormann, F. H., 383
Borrow, A., 184, 185, 186, 187, 188, 189, 190, 207, 210
Borsook, H. C., 87
Borthwick, H. A., 375, 376, 378, 379, 395, 397, 398, 399
Bose, J. C., 250
Bosemark, N. O., 161
Boss, G., 271
Boswell, J. G., 439
Bosz, J. L. Q., 280
Boubals, D., 224
Boulter, D., 431, 432
Bourdeau, P., 384
Bourdouil, C., 406
Bourne, B. A., 285
Bourne, E. J., 41
Bourquelot, E., 434
Bouthillier, L. P., 73, 83, 92
Bowen, H. J. M., 53
Boyd, J. D., 240
Boyer, P. D., 444
Boyko, H., 380
Boynton, D., 68, 88
Boysen-Jensen, P., 154, 155, 158, 164, 168, 171, 176
Bracco, M., 418
Bracken, A., 83
Bradley, D. F., 138, 148
Bramesfeld, B., 71
Brandes, E W., 281, 293, 296
Brannam, B. L., 341
Brannon, M. A., 315
Brase, K. D., 217, 221
Brasil Sobr, M. O. C., 43
Braun, A. C., 417
Brauner, L., 176, 177
Breazeale, E. L., 35, 380
Breazeale, J. F., 380
Bresky, A., 310, 318
Brett, P. G. C., 292, 293
Brian, P. W., 413-26; 184, 185, 186, 187, 188, 189, 190, 194, 195, 196, 197, 198, 199, 200, 201, 205, 206, 207, 208, 210, 400, 413, 415, 416, 417, 421
Bridel, M., 406
Brierley, J. K., 36
Briggs, G. E., 11-30; 11, 13, 18, 22, 26, 146, 205
Briggs, L. J., 239, 257
Briggs, W. R., 447
Brilliant, A. W., 264
Brimhall, B., 406, 409
Britten, R. J., 66, 96, 102, 104, 105, 340
Britton, G., 156
Broadbent, D., 97
Brodie, H. W., 275-308; 296, 297
Brody, S. S., 120, 125, 126, 133
Broeg, C. B., 302
Brohult, S., 66
Brooks, F. A., 314
Brouwer, R., 37
Brown, A. H., 137
Brown, J. C., 31, 42, 49, 50, 58, 434, 437
Brown, J. W., 48, 58
Brown, R., 38, 97
Brown, T. E., 138, 141, 145, 146, 147, 148
Brown, W. H., 257
Broyer, T. C., 53
Brugger, J., 126, 129
Brumfield, R. T., 176, 177
Brunel, J., 309, 322
Brusca, J. N., 48, 56
Bryan, W. H., 431
Bryson, V., 340
Buchholtz, K. P., 383
Budgett, H. M., 239
Bukovac, M. J., 38, 39, 200, 201, 202, 203, 206, 207
Bula, R. J., 386
Bünning, E., 375
Bünsow, R., 202, 206
Burkholder, P. R., 160, 408
Burlet, E., 169
Burlew, J. S., 276, 309, 311, 318, 321, 322, 325, 327
Burnett, W. T., Jr., 42
Burr, G. O., 275-308; 283, 284, 286, 287, 289, 290, 291, 292
Burris, R. H., 42, 50, 84, 93, 95, 96, 163, 444, 446, 447
Burroughs, L. F., 72
Burström, H., 37, 44, 47, 48, 94, 168, 169, 171, 172, 173, 175
Büsgen, M., 237, 250
Businger, J. A., 131
Bustinza, F., 414, 415
Butler, G. W., 12, 15, 24, 97
Butler, R., 407

C

Cain, J. C., 57, 75, 89
Caldwell, D. H., 325
Calhoun, B., 41, 51
Califano, L., 436
Calvin, M., 66, 74, 89, 105, 137, 138, 147, 148, 310, 420, 444
Cameron, J. W., 406, 408
Cameron, S. H., 223, 229
Camus, G., 420
Cannon, H. B., 223
Cantlon, J. E., 377
Capindale, J. B., 142
Capo, B. G., 283, 299, 300
Capoor, S. P., 360
Cardini, C. E., 290
Carey, T. M., 300
Carlone, R., 132
Carpenter, C. W., 286
Carraz, M., 420
Carson, R. S., 145
Carter, H. E., 72
Cartwright, P. M., 38
Castlefranco, P., 445
Catch, J. R., 90, 92
Cathey, M., 78, 89
Cattaneo, A., 182
Cavallini, D., 73
Cercos, A. P., 415
Chaciacarti, A. S., 301
Chaikoff, I. L., 54
Chalmers, R. V., 316
Chambers, L. G., 239
Chance, B., 446
Chandler, B. V., 223, 229
Chandraratna, M. F., 396
Chanley, P. E., 327
Chapman, H. W., 263
Cheadle, V. I., 349-74; 350, 351, 352, 353, 355, 371
Chen, S. L., 146, 361, 361
Chester, V. E., 184, 185, 186, 187, 188, 189, 190, 207, 210
Chiapelli, R., 182
Chibnall, A. C., 65, 66, 97, 104
Childers, N. F., 261, 263, 264, 266
Chilton, S. J. P., 293
Chinloy, T., 282
Chiriboga, J., 290
Chiu, W. F., 182, 184
Cholodny, N., 154, 158, 160, 161, 162, 164, 171, 172, 173
Chu, S. O., 310, 311
Clagett, C. O., 444
Clark, D. G., 244, 250
Clark, L. B., 310, 311, 320
Clark, T. A. B., see Bennet-Clark, T. A.
Clarke, A. E., 401
Clarke, G. L., 387
Clausen, J., 386
Clauss, H., 158, 165
Clayson, D. H. F., 429
Cleland, R., 335
Clements, H. F., 261, 282, 283, 284, 285, 286, 295, 296, 298, 300
Clendenning, K. A., 137-52; 115, 125, 134, 137, 138, 141, 142, 143, 145, 146, 149
Cochran, L. C., 227
Cocking, E. C., 69
Coelingh, W. M., 289
Cohen, L. A., 92
Coker, E. G., 219, 220
Coleman, J. W., 128, 129
Coleman, R. E., 275-308; 293, 301
Coleman, R. G., 75, 86
Collander, R., 335-48; 5, 337, 338, 340
Collyer, D. M., 320
Colwell, R. N., 362
Comar, C. L., 39, 42
Combs, G. F., 327
Commoner, B., 103
Conn, E. E., 433
Connell, G. E., 70
Conrad, C. M., 47, 48, 404
Conrat, H. F., see Fraenkel-Conrat, H.
Constance, L., 385
Contopoulou, R., 445
Convit, J., 328
Conway, E. J., 340
Conway, H. F., 186, 187, 188, 189
Cook, C. W., 257
Cook, P. M., 310, 311, 317, 320, 322
Cooke, D. A., 297
Coop, I. E., 401
Cooper, W. C., 227
Cooper, W. C., Jr., 4, 5
Copeland, E. B., 237, 241, 243, 249, 253
Corkhill, L., 401
Cornelison, A. H., 280, 295, 298, 299
Cortesi, R., 414, 415, 420
Cory, R., 39, 46
Coster, C., 243
Cottam, W. P., 386
Coulson, C. B., 68, 79
Coury, T., 43
Cowan, I. R., 298
Cowie, D. B., 66, 96, 102, 104, 105, 340
Crafts, A. S., 237, 238, 243, 245, 253, 267, 349, 350, 351, 353, 358, 363
Cramer, F., 67
Crane, F. A., 89
Creighton, H. B., 160
Criddle, W. D., 298
Cripps, E. G., 51, 56
Crombie, L., 83
Cromwell, B. T., 443
Crook, E. M., 433
Crooke, W. M., 57, 59
Cropley, R., 217
Cross, B. E., 186, 189, 190, 191, 192, 204
Cross, W. E., 300
Crowdy, S. H., 413, 421
Cruzado, H. J., 301
Currier, H. B., 349-74; 237, 238, 243, 245, 253, 267, 350, 351, 352, 354, 371
Curry, G. M., 197, 198, 202, 206
Curtis, J. J., 408
Curtis, O. F., 244, 250, 370
Curtis, P. J., 184, 185, 186, 187, 188, 189, 190, 207, 210, 415, 416, 417
Curtiss, C. F., 240

D

Dahmen, M., 420
Dalberg, L. Y., 163, 164, 168
Dalton, H. R., 435
Dam, H., 147, 148
Damaschke, K., 145
Damodaran, M., 85
Danielli, J. F., 336, 371
Darling, J. B., 377, 380
Darpoux, H., 415
Darwin, F., 237, 243, 259
Das, N., 171, 172, 175
Das, U. K., 295, 296, 297, 298, 299
Davidson, L. G., 301
Davies, E. E., 37
Davies, J. G., 278
Davies, R. E., 139
Davies, V., 261
Davis, A. R., 5
Davis, B. D., 74
Davis, E. A., 310, 313, 314, 316, 317, 318, 320, 321
Davis, H. C., 327
Davis, J. J., 389
Dawson, C. R., 428, 429, 434, 435, 436, 437
Dawson, R. F., 230
Day, L. H., 227
Day, W. R., 240
Dayton, T. O., 405, 406
De, P. K., 324
De, R., 288
Dearborn, R. B., 205
DeBacker, S. M., 376
DeBruyn, J. A., 56
DeBusk, A. G., 393
Decker, J. P., 384
Dedrick, J., 310, 313, 314, 316, 317, 318, 320, 321
Deerr, N., 276
Defay, R., 240
Degman, E. S., 261
de Haan, H., see Haan, H. de
de Haan, I., see Haan, I. de
de Haan, J. T., see Haan, J. T. de
DeKock, P. C., 40, 49, 58, 59

XUM

de la Roza, J., see Roza, J. de la
Delisle, A. L., 231
Della Bella, D., 414, 415
Delwiche, C. C., 86, 103
DeMoss, J. A., 71
Denffer, D. von, 316, 319
den Honert, T. H. van, see Honert, T. H. van den
Dent, C. E., 67
de Ropp, R. S., see Ropp, R. S. de
DeRover, W., 138, 140, 141, 146
de Stevens, G., see Stevens, G. de
de Stigter, H. C. M., see Stigter, H. C. M. de
de Wet, A. F., see Wet, A. F. de
Dexter, S. T., 257
Dickson, A. G., 230
Dickson, A. Q., 221
Diemair, W., 430
Dillewijn, C. van, 183, 184, 185, 276, 298, 299
Dimond, A. E., 413, 416
Diwald, K. A., 272
Dixon, G. H., 70
Dixon, H. H., 237, 238, 240, 242, 243, 247, 249, 250
Doak, B. W., 401
Dobzhansky, T., 75
Dodds, M. L., 428
Dokhan, R., 86
Done, J., 80, 81, 82
Dorcas, M. J., 4, 5
Döring, H., 316
Doudoroff, M., 74
Dounce, A. L., 98
Downey, M., 340
Downs, R. J., 379
Doyle, W. L., 310
Drawert, H., 335
Drosdorff, M., 48
Dubinina, I. M., 365, 366, 367
Dubnoff, J. W., 87
Dugger, W. M., 264
Dugger, W. M., Jr., 41, 51, 366
Duhamet, L., 169
Duisberg, P. C., 272
Duncan, H. F., 297
Dunegan, J. C., 415, 418
Dunn, F. J., 428, 429
Dunn, G. M., 406
Duranton, H., 77
Durham, L. J., 142
Durrum, E. L., 67
Dusi, H., 415, 418
Dustman, R. B., 237, 238, 250
Du Toit, J. L., 283, 284
Duysens, L. N. M., 115, 126, 127, 128, 132, 137, 141, 143, 144, 146, 147, 148
Dyke, I. J., 168
Dymond, J. A., 53

E

Eaton, F. M., 244, 261
Eberhardt, G., 278
Eberle, G., 173
Ebert, V. A., 163
Ebihara, T., 430
Eckhart, F., 257, 264
Edgerton, C. W., 276
Edmondson, W. T., 387
Edwards, E. T., 184, 185, 199, 210
Eggleston, L. V., 27
Ehrmantraut, H. C., 138, 146, 147, 148
Eiger, I. Z., 435
Eisenberg, A., 318, 322
Ekdahl, I., 168, 173
Elazari-Volcani, T., 247
Elder, C. C., 72
Elder, J. A., 402
Elgabaly, M. M , 34
Elisei, F. G., 182, 183, 184
Ellenberg, H., 385
Elliott, E. S., 187
Ellner, P., 88
Elson, G. W., 194, 195, 197, 198, 199, 200, 201, 206, 207, 210, 415, 416, 417, 421
Elvove, E., 93
Emerson, R., 125, 138, 316
Endlich, B., 335
Enebo, L., 310, 318
Engard, C. J., 281, 293
Englebrecht, L., 85, 88, 89
Eocha, C. O., see O'h Eocha, C.
Epstein, E., 12, 17, 22, 31, 32, 33, 34, 35, 36, 37, 46, 53, 54, 335, 337, 340
Ergle, D. R., 261
Erickson, L. C., 341
Ericson, L. E., 55
Ernster, L., 87, 98
Erxleben, H., 164
Esau, K., 349-74; 243, 350, 351, 352, 353, 355, 358, 359
Eschrich, W., 354, 356, 358
Euler, H. von, 74, 399, 415, 418
Evans, D. F., 119
Evans, H., 281, 283, 284, 285, 295, 296, 297
Evans, H. J., 36, 44, 50, 52, 57, 94
Evenari, M., 318, 320, 321, 322, 327
Evstigneev, V. B., 122, 123
Ewart, A. J., 242, 249
Ewart, G. Y., 298
Eyring, H., 115, 120, 131, 137, 142, 146, 147, 148
Eyster, H. C., 138, 145, 147, 148, 398
Eyster, W. H., 398

F

Faber, E. R., 162, 165, 167
Fager, E. W., 137, 142, 310
Fåhraeus, G., 441
Faivre-Amiot, A., 415
Farnell, R. G. W., 278
Fawcett, C. H., 159
Fawzy, H., 56
Fearon, W. R., 86
Feillafé, S. M., 283, 300
Fel'dman, A. L., 440
Fellows, H., 184, 210
Fennell, D. I., 186, 187, 188, 189
Ferreira, C., 278
Ferrell, W. K., 38
Fiedler, H., 154, 163, 168, 169
Field, C. P., 221
Fife, J. M., 145
Filewicz, W., 227
Filinger, G. A., 227, 228
Fink, H., 327, 328
Finkner, R. E., 402
Finlay, A. C., 419, 420
Fisher, A. W., Jr., 318, 325, 327, 328
Fisher, F. J. F., 379
Fisher, G., 145
Fisher, J. C., 240, 241
Fisher, R. B., 66
Fitting, H., 267
Flesch, P., 437
Floor, J., 229
Florell, C., 36, 44, 46
Fogg, G. E., 31, 45, 66, 93, 309, 310, 320, 324
Folkers, K., 86
Follmann, G., 336
Foltz, C. M., 93
Fong, J., 319
Fonnesu, A., 139
Forbes, A., 286, 287, 291, 292
Forbes, I., 404
Forde, H. I., 229
Forster, L. S., 120
Förster, T., 121
Forster, W. A., 58
Fort, C. A., 280, 301
Fosse, R., 85
Foster, R. F., 389
Foulkes, E. C., 34, 35, 341
Fowden, L., 65, 68, 71, 72, 74, 76, 78, 79, 80, 81, 82, 83, 93
Fowells, H. A., 380
Fowlks, W. L., 436, 438
Fox, S. W., 66, 88
Fraenkel-Conrat, H., 103
Frampton, V. L., 145
Franck, J., 119, 122, 126, 128, 132, 133, 134, 138, 145, 147, 148, 149
Fraps, G. S., 408
Freed, S., 116, 124

XUM

Freeland, E. C., 279
Freeland, R. O., 264
Freiberg, S. R., 88
French, C. S., 121, 126, 137, 143, 146, 310, 313, 314, 316, 317, 318, 320, 321
Frenkel, A. W., 137, 138
Freudenberg, K., 90
Frey-Wyssling, A., 272, 344, 360
Fricke, H., 340
Fried, M., 43
Friedrich, G., 224
Friesner, R. C., 246
Friloux, J. J., 289
Frisch, A. W., 145
Fritsch, F. E., 138
Fritts, E. C., 246
Fritts, H. C., 246
Fritz, G., 432, 445
Frohardt, R. P., 72
Frontali, G., 73
Fuhriman, D. K., 298
Fujii, M., 290
Fujimori, E., 123
Fujimura, K., 146
Fujiwara, A., 52, 310
Fukunaga, K., 199, 200, 201, 204, 207
Fukuoka, F., 210
Fuller, R. C., 147
Furth, R., 240
Fusari, S. A., 72

G

Gabellini, C., 414, 415
Gaffron, H., 115, 119, 137, 148, 309
Gale, E. F., 66
Galinat, W. C., 395, 396
Galston, A. W., 42, 50, 54, 162, 163, 164, 168, 398, 446
Gansa, A. N., 88
Garavel, L., 225
Gardell, S., 145
Gardner, D., 421
Garner, R. J., 222, 224, 225, 228
Garrard, A., 171
Gartler, S., 75
Gasser, R., 269
Gast, A., 166, 167
Gauch, H. G., 31-64; 41, 47, 51, 261, 366
Gäumann, E., 188, 413, 415, 417
Gavard, R., 441
Gavrilova, V. A., 123
Geerts, J. M., 300
Geiger, R., 376
Geiger-Huber, M., 169
Geissman, T. A., 404, 405, 406
Genuth, S. M., 71
Geoghegan, M. J., 309, 310 313, 314, 317, 321, 322, 327
Geraldson, C. M., 46
Gerdes, G., 316
Gerhardt, F., 404
Gerloff, G. C., 55
Gibbs, R. D., 238, 240, 242, 243, 244, 249, 250
Gierer, A., 103
Giese, A. T., 126
Gifford, E. M., Jr., 350, 353, 355
Gill, R. F., 278, 280, 281
Gilmour, H. S. A., 147, 149
Gilvarg, C., 74
Girard, R., 414, 415, 420
Giri, K. V., 86, 93
Glass, B., 44, 51, 52, 66, 93, 97
Glass, H. B., 50
Glazener, M. R., 73
Goddard, D. R., 427, 433
Goedheer, J. C., 121, 125
Goldacre, P. L., 446
Goldacre, R. J., 371
Goldschmidt, P., 342
Golueke, C., 326, 327
Gondo, A., 292
Gonzáles, F. A. Z., 276
Good, N. E., 162
Good, R., 385
Gooding, E. G. B., 288
Goodman, R. N., 415, 418
Goodwin, R. H., 164, 167, 173
Goodwin, T. W., 419
Gordon, S. A., 158, 159, 160, 162, 163, 164, 447
Gordon, W. C., 404
Gordon, W. L., 183, 184
Gorham, P. R., 142, 143, 145, 146, 397
Gorter, C. J., 154, 158, 160, 161, 162, 163, 196
Gortner, R. A., 143
Gortner, W. A., 163, 446
Goss, J. A., 203, 206
Gotaas, H. B., 310, 311, 317, 326, 327
Gottesman, E., 318, 320
Gottlieb, D., 415
Graber, R. P., 86
Graf, E., 355
Graham, B. F., Jr., 388
Graham, J.-R., 310, 311, 317, 321
Graner, E. A., 43
Granfield, C. O., 261
Granick, S., 137, 138
Grant, R., 301
Grant, V. Q., 164
Graves, G., 217
Graves, H. A., 227
Gray, R., 383
Gray, R. A., 415, 416, 421
Green, D. E., 52, 94
Green, L. F., 429
Greene, S., 164, 167, 173
Greenidge, K. N. H., 237-56; 240, 244, 247, 248, 251
Gregg, D. C., 440
Gregory, F. G., 104, 229
Gries, G. A., 415
Griffith, R. B., 408
Griffiths, L. A., 419
Grisebach, H., 147
Grob, E. C., 407. 418, 419
Grobbelaar, N., 65, 71, 72, 75, 76, 82, 90, 92
Gross, C. R., 68, 88
Grove, J. F., 189, 190, 192, 421
Grubb, N. H., 221, 227
Grumbein, M. L., 166
Grümmer, G., 415
Grundmann, A. W., 388
Guernsey, F. S., 161, 162, 169
Guggenheim, M., 70
Gummert, F., 317, 318, 319, 321
Gunslaus, I. C., 74
Gupta, N. C. D., 280
Gurgel, J. T. A., 223, 229
Gustafson, F. G., 37, 38, 40
Györgyi, A. S., see Szent-Györgyi, A.

H

Haagen-Smit, A. J., 159, 164, 205
Haan, H. de, 196
Haan, I. de, 196
Haan, J. T. de, 184, 185, 199
Haas, A. R., 3
Haas, A. R. C., 48, 56
Haas, E., 443
Haas, V. A., 310
Haasis, F. W., 246
Hace, E., 146
Hackett, D. P., 427, 428, 432, 443
Hackney, F. M. V., 437
Hada, N., 143
Haertl, E. J., 49
Hagberg, A., 404
Hageman, E., 70
Hagen, C. E., 34, 35, 53
Hahn, H., 266
Haider, K., 239
Haines, F. M., 242, 380
Halais, P., 282, 283, 295
Haldane, J. B. S., 394
Hall, A., 58, 59
Hall, A. G., 310
Hall, N. S., 52, 57
Halvorson, H. O., 100
Hamilton, K. C., 383
Hammond, L. C., 31
Hamner, C. L., 415, 418, 419
Hamner, K. C., 377
Hance, F. E., 282
Handley, R., 36, 47, 56

Handley, W. R. C., 251
Hanes, C. S., 70, 98
Hanly, V. F., 29
Hansen, B. A. M., 164, 174
Hansen, G. A., see Asboe-Hansen, G.
Hansen, H. N., 183
Hansen, O. H., see Holm-Hansen, O.
Hansen, O. R., 415
Hansen, R. G., 70
Hansford, C. G., 292
Hansl, N. R., 431
Hanson, J., 23, 33, 34
Hara, M., 42, 49
Harder, R., 202, 206, 309, 319, 320, 322
Hardin, G. J., 310, 317, 319
Hardy, F., 280, 281
Harer, L., 396
Harkens, W. D., 238
Harley, J. L., 36
Harmon, F. N., 229
Harrer, C. J., 443
Harris, E. S., 3, 8
Harris, G., 67, 68, 81
Harris, H. C., 39
Harris, R., 399
Harris, W. E., 420
Hartree, E. F., 137, 427, 437, 438, 443, 447
Hartt, C. E., 275-308; 278, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 298, 299, 300
Harvey, E. N., 239, 344
Hase, E., 310, 311, 312, 313, 314, 315, 316, 318
Hashimoto, T., 198, 200, 207
Hashioka, Y., 184
Haskell, T. H., 72
Haskins, C. P., 311
Haskins, F. A., 439
Hassan, H. H., 415
Hassan, H. M., 230
Hatcher, E. S. J., 228
Hatt, J. L., 86
Hatton, R. G., 217, 218, 219, 220, 222
Hauge, S. M., 444
Hauser, G., 278
Havinga, E., 420
Hawker, L. E., 154, 171
Haxo, F. T., 138, 140, 146
Hay, J. R., 365
Hayashi, T., 186, 188, 189, 194, 195, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 210, 421
Hayes, A. D., 340
Hayward, H. R., 34, 53
Heald, W. R., 41, 53
Hearman, J., 218
Hearn, W. R., 72
Heathcote, J. G., 408
Hegarty, M., 86, 92
Heil, H., 271
Heinicke, A. J., 264
Heinze, P. H., 397
Heller, L., 418
Hellstrom, H., 399
Hemmi, T., 182, 183, 184, 186, 207
Hemming, H. G., 184, 185, 186, 187, 188, 189, 190, 194, 195, 197, 198, 199, 200, 201, 205, 206, 207, 208, 210, 400, 415, 416, 417, 421
Henderson, J. H. M., 404
Hendricks, S. B., 37, 375, 376, 378, 395, 397, 398, 399, 402, 434
Henehan, C., 184, 185, 186, 187, 188, 189, 190, 207, 210
Hengeveld, B., 280
Henry, A. W., 415
Heppel, L. A., 443
Hepton, C. E. L., 353
Hernandez, M. G., 74
Herold, E., 328
Herrero, J., 223, 224, 225, 226
Herschberg, L. H., see Heymann-Herschberg, L.
Hervey, A., 418, 419
Hervey, R. J., 420
Herzfeld, K. F., 149
Hes, J. W., 290, 293, 301
Hewetson, F. N., 223
Hewitt, E. J., 44, 52
Heymann-Herschberg, L., 50
Hiesey, W. M., 386
Hietala, P. K., 71, 82
Highkin, H. R., 398
Higinbotham, N., 23, 33, 34
Higuchi, T., 442
Higuti, T., 183
Hilkenbäumer, F., 217, 220
Hill, R., 137, 143, 144, 147, 427, 437, 443, 447
Hill, S. E., 8
Hillman, W. S., 42, 50
Hinreiner, E., 404
Hinsvark, O. N., 87
Hinton, J. J. C., 408
Hirao, H., 146
Hirata, K., 183
Hirata, Y., 72
Hirayama, S., 187
Hird, J. F., 98
Hirs, C. H. W., 69
Hirschfelder, J. O., 240
Hixon, R. M., 406
Hoagland, D. R., 5, 361
Hoblyn, T. N., 217, 227
Hodge, J. A., 139
Hodgson, G. L., 384
Hodgson, H. J., 386
Hoffhine, C. E., 86
Höfler, K., 257, 269, 271, 343
Hofmeister, L., 338, 345
Hogan, G. L., 138
Hogness, T. R., 433
Holden, J. T., 74, 90
Holdheide, W., 355
Holle, H., 237, 242
Holley, R. W., 75, 89
Holly, F. W., 86
Holm, G., 441
Holman, R. T., 444, 445
Holmberg, D. M., 50
Holmes, F. O., 359
Holmes, R. S., 42, 49, 50, 58
Holmgren, R. C., 385
Holm-Hansen, O., 55
Holt, A. S., 116, 128, 129
Holzer, H., 316
Homan, C., 238
Honda, S. I., 36, 38, 429, 431, 432
Honert, T. H. van den, 34, 35, 283, 285, 370
Honig, P., 280
Honing, J. A., 397
Hood, D. W., 87
Hood, S. L., 147, 148
Hooymans, J. J. M., 34, 35
Hope, A. B., 12, 20, 22, 27, 37, 139
Hopkins, F. G., 433
Hopkins, H. T., 32, 35, 37
Horecker, B. L., 74, 105, 443
Hori, S., 182, 183, 194, 198, 202, 203
Horié, K., 337
Horiuchi, M., 199, 200, 201, 204, 207
Horowitz, N. H., 86, 90, 393, 394
Horricks, J. S., 415
Housley, S., 155, 156, 159
Houwink, A. L., 344
Howard, W. H., 397
Huber, B., 243, 253, 352, 355, 381
Huber, M. G., see Geiger-Huber, M.
Hughes, C. G., 276
Huglin, P., 224
Hulbert, L. C., 385
Hull, H. M., 363
Hulme, A. C., 76, 90
Hülsbruch, M., 357
Hulsmann, B., 220
Humbert, R. P., 281, 284
Humphreys, M. H. K., 377, 380
Humphreys, T. E., 41, 51
Hungate, R. E., 237, 244
Hunter, J. G., 59
Hurst, L. A., 300
Hurwitz, J., 105
Hutinel, P., 418
Hutner, S. H., 311, 418
Hylmö, B., 12, 32, 37, 422

I

Ichioka, P. S., 44, 52, 310

Igarashi, H., 189, 191, 192, 199, 207
Ikeya, J., 182
Iljin, W. S., 257-74; 257, 258, 259, 260, 261, 262, 263, 265, 266, 267, 269, 270, 271
Imura, J., 186, 187, 194, 197
Inada, Y., 146
Inagaki, C., 278
Innes, R. F., 282, 283, 298
Irreverre, F., 92
Irving, A. A., 146
Irwin, M., 5, 7
Ishai, R. B., see Ben-Ishai, R.
Isherwood, F. A., 73, 98
Ishibashi, S., 313, 317, 320
Ishikawa, Y., 145
Ishizuka, K., 201, 207
Islam, M. A., 39
Ito, R., 325, 327
Ito, S., 181, 182, 183, 184, 186, 187, 198, 199, 203, 207
Ito, T., 72
Iwamura, T., 146, 310, 311, 312, 313, 314, 315, 316, 318, 320
Iyengar, M. R. S., 415, 420

J

Jaag, O., 415, 417
Jackson, R. W., 186, 187, 188, 189
Jackson, W. R., 278
Jacobs, E. E., 116
Jacobs, M. H., 344
Jacobson, L., 33, 34, 47, 49, 56, 57
Jacques, A. G., 4, 5, 6, 7
Jagendorf, A. T., 66, 105, 139, 140
James, W. O., 237, 364, 427, 430, 431, 432, 437
Janes, B. E., 380
Jannes, L., 74
Jeffreys, E. G., 184, 185, 186, 187, 188, 189, 190, 207, 210, 417
Jenkins, J. A., 397, 398, 407
Jennings, D. N., 36
Jensen, P. B., see Boysen-Jensen, P.
Jerchel, D., 158, 159
Jirovec, O., 418
Jodan, N. E., 407
Joham, H. E., 56
Johannssen, D. W., 72
Johnson, B. L., 405, 406
Johnson, C. M., 53
Johnson, F. D., 38
Johnson, M. P., 99, 157, 336
Johnson, S. W., 428
Johnston, J. A., 74, 310
Johnstone, J. H., 76, 90
Joly, J., 237, 243
Jones, D. B., 278
Jones, E. W., 44, 52
Jones, E. W. B., see Bolle-Jones, E. W.
Jones, H. A., 401
Jones, J. W., 408
Jones, L. H. P., 57
Jones, M. E., 87
Jones, R. L., 73, 176
Jones, S. S., 40
Jones, T. S. G., 90, 92
Jönsson, E., 156, 161, 163, 164, 170, 171
Jorgensen, E. C., 405, 406
Jorgensen, J., 328
Jorpes, J. E., 145
Joselow, M., 429
Jost, L., 162, 167, 171, 172
Joubert, T. G., 404
Juhren, M., 375
Julander, O., 261
Juni, E., 74

K

Kahl, H., 271, 272
Kahn, A., 203, 206
Kaiser, E., 280
Kaiser, S., 157
Kaji, J., 90, 188
Kalnitsky, G., 74
Kambayashi, T., 183, 210
Kamen, M. D., 137, 138
Kamerling, S. E., 6
Kamiya, N., 339
Kanabe, S., 186, 188
Kandler, O., 169, 172, 173, 175
Kantor, J. H., 396, 398
Kanwisher, J. W., 238, 239, 240, 242, 244, 247, 248, 249, 253
Kapil, Y. P., 279
Kargl, T. E., 407
Kari, S., 90, 92
Karling, J. S., 246
Karnatz, H., 227
Karschon, R., 378, 386
Kasha, M., 119
Kasting, R., 86
Kastle, J. H., 93
Katagiri, M., 74
Katayama, E., 187
Katayama, T., 143
Kates, M., 146
Kato, J., 195, 197, 198, 199, 201, 205, 206, 207, 208, 421
Kato, Y., 195, 199, 203, 206, 207
Katyal, S. L., 217
Katznelson, H., 74
Kavanagh, F., 413
Kawamura, I., 442
Kawarada, A., 186, 187, 188, 189, 190, 191, 192, 193, 194, 204, 207, 210
Kay, R. E., 398
Ke, B., 146
Kearney, E. B., 428
Keck, D. D., 386
Keever, C., 383
Kefford, J. F., 223, 229
Kefford, N. P., 154, 155, 156, 159, 168
Kehr, A. E., 408
Keilin, D., 429, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443
Keitt, G. W., 415
Keller, V., 419
Kelli, A. C., 227
Kelly, F. H. C., 280, 281
Kemmer, E., 227
Kemmerer, A. R., 408
Kendall, W. A., 39, 363, 364
Kenjo, M., 283, 284, 285, 286
Kenrick, F. B., 239
Kent, M., 163, 446
Kenten, R. H., 443, 444, 446, 447
Kern, H., 337, 415
Kernan, R. P., 340
Kértesz, D., 436
Kertesz, Z. I., 48
Kessler, E., 147
Ketchum, B. H., 309, 310, 320, 322
Khalil, A., 164
Khanna, K. L., 290, 291, 301
Kidd, F., 205, 223
Kienholtz, J. R., 415, 418
Kimura, J., 181, 182, 183, 184, 198, 199, 203
Kimura, K., 183
King, A. L., 239
King, C. G., 429
King, F. E., 90, 92
King, K. W., 84
King, N. J., 276
King, R. L., 142
King, T. J., 90, 92
Kinzel, H., 143, 342, 344
Kirchhoff, R. H., 223, 226, 228
Kirpal Singh, K., 223, 226
Kitagawa, M., 86
Kitamura, H., 186, 187, 188, 189, 190, 191, 192, 193, 194, 204, 207, 210
Kittredge, J., 381
Klackle, R. L., 222
Klein, G., 85
Klemmer, H. W., 415
Klemperer, F., 429
Knapp, R., 385
Knight, C. A., 103
Knight, R. C., 219, 221, 231
Kobayashi, G., 86, 90
Kobayashi, Y., 74
Kobel, H., 188, 415
Kobiakova, A., 261
Koehler, B., 199, 210
Koepfli, J. B., 162, 164, 168, 169
Kofler, H., 342

Kofranek, A. M., 206
Kögl, F., 159, 164
Köhler, E., 359, 360
Koizumi, C., 200, 207, 208
Kok, B., 131, 310, 311, 312, 313, 314, 315, 316, 317, 319, 320, 322
Kolbe, R. W., 352
Kolkunov, W., 269
Koller, D., 375, 376
Koningsberger, V. J., 289
Konishi, T. A., 181
Kortschak, H. P., 275-308; 289
Koski, V. M., 398
Kosobutskaya, L. M., 125
Kostermans, D. G. F. R., 159
Kovachevich, R., 74
Kowkabany, G. N., 280, 281
Kozlowski, T. T., 261, 263, 384
Kral, F., 341
Kramer, H. H., 406
Kramer, P. J., 38, 40, 139, 238, 250, 257, 335, 346, 384
Krasilnikov, N. A., 413, 415
Krasnoselski-Maximov, T. A., 257
Krasnovskii, A. A., 123, 125
Kratz, W. A., 53, 138, 310, 312
Krauss, R. W., 45, 49, 56, 310, 311, 317, 319, 320, 322
Krebs, H. A., 27
Krotkov, G., 102, 104, 105
Kruckeberg, A. R., 381, 382, 386
Krumholz, L. A., 389
Krupnikova, T. A. M., see Malkina-Krupnikova, T. A.
Kubota, T., 298
Kubowitz, F., 434, 436, 438, 439
Kugler, I., 396, 397
Kuhn, H., 121
Kuijper, J., 297
Kulka, D., 74
Kumm, J., 143
Kun, E., 74
Kunkel, L. O., 360
Kuntz, J. E., 243, 253
Kuprevich, V. F., 366
Kuraishi, S., 198, 200, 207
Kurosawa, E., 182, 183, 184, 185, 186, 187, 194, 195, 197, 198, 199, 200, 202, 207
Kursanov, A. L., 362, 364, 365, 366, 367, 368, 369, 371
Kurtz, E. B., 404
Küster, E., 354

L

Lacharme, J., 418, 419
Lack, L., 58
Lagasse, F., 48
Lagaude, V., 218
Laibach, F., 403
Laine, T., 65
Lakshminarayan, K., 417
Lal, K. N., 31, 288, 290, 301
Lambertz, P., 352
Lamm, R., 196
Lampitt, L. H., 429
Lance, C., 420
Landrau, P., Jr., 34, 282, 300
Landsford, E. M., 72
Lane, R. H., 164, 166
Lang, A., 190, 195, 202, 203, 206, 207, 209, 396, 406
Langford, C. T., 186, 187, 188, 189
Langham, D. C., 408
Langridge, J., 397, 398, 402
Lansing, A. I., 35
Lardy, H. A., 43
Larsen, E. C., 386
Larsen, H., 138
Larsen, N., 281, 293
Larsen, P., 163, 176
Larson, K., 389
Laser, H., 443
Lasher, A., 316
Latimer, P., 120, 125, 126, 129
Latus, M., 418, 419
Laughnan, J. R., 404, 406
Lauritzen, J. I., 297, 301, 302
Lazenby, A., 385
Lazo, F., 285
Leach, J. H., 396
Leavenworth, C. S., 65, 76
Leben, C., 415
Lederer, E., 66
Lederer, M., 66
Ledon, A. C., 276
Lee, H. A., 301
Leggett, J. E., 12, 17, 32, 33, 35, 37, 46, 53, 54
Legrand, G., 441
Leloir, L. F., 290
Leme, J., Jr., 223, 229
Leonian, L. H., 183, 185, 315
Leopold, A. C., 161, 162, 169
Le Rosen, A. L., 407
LeStrange, R., 69
Levan, A., 165, 172
Leupold, U., 393, 394
Levitt, J., 238, 271, 272, 335
Lewin, J. C., 36, 55
Lewis, C. M., 125, 138
Lewis, M. S., 73
Lewis, S., 434, 435
Lexander, K., 155, 156, 159
Leyerer, G., 264
Li, K., 340
Li, P. L., 438
Libbert, E., 163
Liebig, J., 84, 85
Lillick, L., 310, 322
Lilly, V. G., 315
Linck, A. J., 363
Lincoln, R. E., 407
Lindberg, O., 87, 98
Lindberg, R. G., 389
Lindeberg, G., 441
Lindemann, R., 387
Lindsey, A. A., 377
Lindstrom, E. W., 404
Linehan, P. A., 401
Lingle, J. C., 50
Link, K. P., 401
Linko, P., 87, 93
Linn, M. B., 415
Linsbauer, K., 258
Linschitz, H., 117, 118, 122, 124, 132
Linser, H., 85, 157, 165
Lintner, J., 283
Lioret, C., 77
Lipman, F., 87
Lippich, F., 88
Lippincott, J. A., 103
Little, T. M., 396, 398, 401
Liverman, J. L., 395, 396
Livingston, B. E., 244, 257
Livingston, R., 116, 117, 118, 119, 120, 121, 122, 123, 124
Lloyd, P. B., 184, 185, 186, 187, 188, 189, 190, 207, 210
Lockhart, J. A., 197, 200, 204, 207
Lockingen, L. S., 393
Lockwood, J. L., 415
Loeb, J., 2, 3
Löhnis, M. P., 56
Lona, F., 195, 198, 202, 203, 206, 207, 208
Loneragan, J. F., 52
Long, F. A., 40
Longley, R. P., 223
Loomis, W. D., 94
Loomis, W. E., 31, 239, 263
Loosanoff, V. L., 319, 327
Lopez, A. C., 414, 415
López, M. A. L., see Lugo-López, M. A.
Lotfy, T., 165, 172
Lott, J. R., 335
Loustalot, A. J., 263, 301
Love, W. E., 238, 239, 240, 242, 244, 247, 248, 249, 253
Lowe, D., 417
Lowy, P. H., 74, 90
Lubin, G., 244
Luckwill, L. C., 217
Ludwig, B. J., 436
Ludwig, H. F., 310, 311, 326
Lugg, J. W. H., 66
Lugo-López, M. A., 282, 300, 301
Luke, H. H., 409
Lumry, R., 115, 120, 130, 131, 133, 141, 142, 144, 145, 146, 147, 148, 149
Lund, E. J., 251

Lundberg, W. O., 444
Lundegardh, H., 11, 20, 36, 147, 156, 166, 167, 168, 173, 240, 243, 244, 249, 252, 253
Lunt, O. R., 49
Lüttgens, W., 138
Lutz, H. J., 240
Lwoff, A., 418
Lyman, C. M., 87
Lynch, L. J., 223, 229
Lynch, V. H., 74, 89, 129, 310, 311, 326, 420
Lyon, A. G., 397
Lyon, H. L., 297
Lyttleton, J. W., 106

M

McAleer, W. J., 310, 317, 322
McCarthy, F. J., 429
McClendon, J. H., 139, 140, 141, 143, 438
McClintock, J. A., 222
McCollum, G. D., 408
McCready, C. C., 36
McDermott, J. J., 244
MacDougal, D. T., 240, 243, 245, 250
Macek, T. J., 278
McElroy, W. D., 31, 42, 44, 45, 51, 52, 54, 66, 93, 95, 97, 239
MacGee, J., 74
McGeorge, W. T., 35, 380
McGlynn, S. P., 119
McGregor, S. M., 159, 170
Mach, F., 415
Macht, D. I., 166
McIlrath, W. J., 51, 56
McKaig, N., 300
McKee, H. S., 76
McKenzie, D. W., 219, 220, 221, 231
Mackie, W. Z., 43
Mackinney, G., 397, 398, 407
McLane, J. W., 257
McLean, E. O., 34
McLean, F. T., 288, 407
McLean, J. D., 139
Macmillan, A., 341
McMillan, C., 381, 382, 386
MacMillan, J., 189, 190, 192
MacNab, J., 414, 415, 420
McQuistan, M., 407
McRary, W. L., 263, 272
McRorie, R. A., 73
McVeigh, I., 408
Magaldi, A., 74
Magasanik, B., 74
Magee, R. J., 428, 435
Magee, W. E., 95
Magness, J. R., 261
Maguire, W. P., 377
Mahler, H. R., 52, 94, 430
Maitlen, G., 415
Maizel, J. V., 39
Makisumi, S., 86
Malavolta, E., 43, 223, 229
Malins, M. E., 244
Malkina-Krupnikova, T. A., 264
Mallette, M. F., 434, 435, 436, 437
Mancini, P., 105
Mandels, G. R., 183, 186
Mangelsdorf, A. J., 275
Mangelsdorf, P. C., 408
Mann, P. J. G., 443, 444
Mann, T., 429, 434, 435, 436, 437, 439, 440, 441, 442
Manning, D. T., 163
Mapson, L. W., 433, 434
Marak, R. R., 327
Margolis, D., 68, 88
Markh, A. T., 440
Markle, J., 361, 362
Marlatt, R. B., 415
Marmer, D., 164
Marsh, G. L., 223, 229
Martell, A. E., 49
Marth, P. C., 194, 203, 206, 207, 208, 210
Martin, D. C., 38
Martin, J. P., 276, 281, 284, 285, 286
Martin, L. F., 280
Martin, R. P., 377, 385
Martyn, E. B., 182
Maschek, F., 157
Maskell, E. J., 264
Mason, H. L., 381, 383
Mason, H. S., 436, 438, 440, 442
Mason, T. G., 370
Mathews, M. B., 433
Mathur, P. S., 301
Matikkala, E. J., 72
Matlow, S. L., 116
Matsuda, J., 280
Matsumoto, H., 75, 183, 184
Matsunaka, S., 200, 207
Matz, J., 293
Maximov, N. A., 257, 267
Maximov, T. A. K., see Krasnoselski-Maximov, T. A.
Maxwell, W., 297
Mayer, A. M., 318, 320, 321, 322, 327
Mayer, H., 267
Mazelis, M., 42, 43, 47, 49, 50, 55, 56
Meagher, W. R., 44, 52, 53, 57
Means, T. H., 381
Meek, E. S., 121, 127
Mees, G. C., 200
Meesters, A., 164, 173
Meeuse, B. J. D., 427
Meffert, M.-E., 310, 316, 317, 318, 319, 320, 321, 322
Meier, R. L., 309
Meiss, A. N., 76
Meister, A., 73, 74
Melin, E., 388
Melville, J., 85, 88, 401
Menke, G., 125
Menke, W., 125
Menzel, R. G., 41, 53
Mercer, F. V., 25, 139
Mercer, S. P., 401
Mertz, E. T., 75
Metcalfe, T. P., 176
Meyer, B. S., 139, 250
Meyer, J., 239
Meyer, K., 257
Meyer, K. H., 20
Michaels, H., 138, 145
Michelson, C., 94
Middleton, L. J., 36
Miescher, G., 415
Miettinen, J. K., 67, 74, 76, 84, 87, 92, 104
Migsch, H., 269
Millar, F. K., 98
Miller, E. C., 238, 243, 249, 250, 267, 271
Miller, G. W., 36, 58
Miller, L. L., 75
Miller, R., 381
Miller, R. L., 415
Miller, S. L., 66
Miller, W. H., 440
Millerd, A., 427
Milner, H. W., 137, 143, 146, 261, 309, 310, 313, 314, 316, 317, 318, 319, 320, 321
Mingioli, E. S., 74
Mitchell, H. K., 393, 394
Mitchell, J. E., 189, 207
Mitchell, J. W., 41, 194, 203, 204, 206, 207, 208, 210, 264
Mitsui, T., 280
Mituya, A., 310, 311, 312, 313, 314, 315, 318
Miyachi, S., 316
Modi, V. V., 419
Modlibowska, I., 227
Moewus, F., 156, 165
Moewus, L., 156, 165
Moisio, T., 92
Molisch, H., 250, 261
Mommaerts, W. F. H., 429
Monk, R. J., 51
Monroe, R. A., 42
Montenegro, H., 223, 229
Montfort, C., 261, 263, 266
Montgomery, C. M., 84
Montgomery, H. B. S., 227
Monty, K. J., 98
Moog, F., 371
Moon, P., 314
Moore, M. H., 227, 228
Moore, S., 68, 69
Morel, G., 77
Moreland, C. F., 293
Morgan, D. G., 200
Moriguchi, S., 284, 285, 286
Morimoto, I., 72, 442

Morimura, Y., 315, 316, 318, 319, 320, 322, 323, 328
Morris, C. J., 71, 72
Morris, M. P., 73, 97
Morris, R. F., 251
Morris, W. T., 415, 418
Morrison, M., 98
Mortimer, D. C., 73
Mosse, B., 224, 225
Mostafa, M. A., 199
Mothes, K., 85, 88, 89
Motoyama, H., 187
Mourgue, M., 86
Moustafa, E. M., 433
Moutschen, J., 420
Moyer, D., 408
Moyse, A., 319
Mozingo, R., 86
Mueller, R. T., 49, 229
Muir, R. M., 208
Mukherjee, S., 279
Mulder, E. G., 52
Mulholland, T. P. C., 189, 190, 192
Muller, C. H., 383
Müller, E., 86
Müller, H., 221
Müller, R., 164, 165
Müller, R. S., see Staab-Müller, R.
Muller, W. H., 383
Münch, E., 237, 250, 351, 370
Munger, H. M., 400, 401
Mungomery, R. W., 276
Munsell, H. E., 278
Murakami, Y., 194, 197, 198, 199, 200, 201, 203, 206, 207, 208, 421
Murayama, N., 188, 200, 201, 204, 206
Murneek, A. E., 205
Murphey, H. C., 402
Müssle, L., 375
Musso, H., 66
Muzik, T. J., 301
Myers, J., 53, 138, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 325

N

Naef-Roth, S., 188, 415
Nagao, K., 278
Nagao, M., 154, 158, 161, 165, 168
Nakabayashi, T., 143
Nakanishi, K., 72
Nakayama, K., 400
Nakayama, T., 184
Narasimhan, R., 292, 293
Nason, A., 42, 44, 45, 52, 54, 93, 94, 439
Naude, C. J., 229
Naundorf, G., 161, 165
Naylor, A. W., 84, 169, 170, 395, 396
Nazarova, P., 267, 269
Neely, J. W., 404
Neger, T. W., 261
Nelson, C. D., 51
Nelson, E. M., 278
Nelson, G. E. N., 191
Nelson, J. M., 434, 435, 437, 439
Nelson, S. H., 223
Nestel, L., 37, 76
Nestler, H. A., 157
Nétien, G., 418, 419, 420
Newcomb, E. H., 431
Newman, F. H., 238
Newman, J. E., 377
Newmark, P., 103
Newton, A. C., 408
Newton, R. A., 272
Ng, M. J., 103
Nicholas, D. J. D., 45, 52, 94
Nickell, L. G., 414, 419, 420
Nicolin, P., 225
Nielsen, E. S., 310
Nielsen, N., 164
Nielsen, T. R., 33, 34, 46, 56
Nienow, I., 415, 419, 421
Nieva, F. S., 158, 160
Nightingale, G. T., 38, 47, 76, 94
Nihei, T., 146, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 322
Nilsson, H., 388
Nisikado, Y., 182, 183, 184, 185
Nitsch, C., 206, 208
Nitsch, J. P., 70, 76, 82, 206, 208
Niu, C. I., 103
Nixon, I. S., 184, 185, 186, 187, 188, 189, 190, 207, 210
Noack, K., 138, 145
Noll, C. R., Jr., 444
Nord, F. F., 278
Nordenhausen, M., 244
Norman, A. G., 413, 415, 416
Norris, G. L. F., 184, 185, 186, 187, 188, 189, 190, 207, 210
Norris, L., 138, 420
Norris, P., 140
Norris, R. E., 138
North, C. P., 40
Norton, R. A., 38
Novak, R., 95
Novelli, G. D., 71
Nutman, F. J., 258
Nyunoya, T., 318

O

Obata, Y., 145
Oberlander, G. T., 381
Ochoa, S., 74, 94, 444
Ochs, G., 153, 165, 166
Odum, E. P., 387, 388, 389
Odum, H. T., 387, 388, 389
Oertli, J. J., 49, 57
Ohashi, M., 72
O'h Eocha, C., 140
Ohki, K., 53, 54, 55
Oland, K., 68
Oldén, E. J., 227
Oleson, J. J., 310, 321
Olmsted, C. E., 377, 386
Olsen, C., 40
Ono, K., 210
Onslow, M. W., 436, 437, 439
Oorschot, J. L. P. van, 310, 311, 312, 313, 314, 316, 317, 319, 320, 322
Ooyama, N., 383
Ordin, L., 33, 34, 58, 335
Ory, R. L., 87
Oster, G., 124
Osterhout, W. J. V., 1-10; 1, 2, 3, 4, 5, 6, 7, 8
Österlind, S., 310
Ostrovskaia, M., 267, 269
Osvald, H., 383
Oswald, W. J., 310, 311, 317, 326, 327
Otake, S., 183, 210
Otis, C. H., 244
Otsuki, T., 187, 188
Ouellet, C., 74, 89
Oura, E., 342
Overbeek, J. van, 168, 208, 231, 281, 296, 349, 399
Overstreet, R., 33, 34, 36, 46, 47, 56
Overton, E., 7
Overton, J. B., 240, 243, 245, 250
Owen, R. D., 393
Owens, K. E., 121
Ozanne, P. G., 50

P

Pacheco, J. A. C., 43
Padmanabhan, S. Y., 183, 184
Padwick, G. W., 182
Pagan, C., 73, 97
Page, A. C., Jr., 72
Paleg, L. G., 447
Palmer, K. S., 2
Palser, B. F., 51
Pankratova, N. M., 361
Pappenheimer, J. R., 344
Pariser, R., 122, 123
Parish, D. H., 280
Parker, J., 270
Parker, M. W., 376, 378, 398
Parthasarathi, N., 399
Parthasarathy, S. V., 300
Passecker, F., 225
Pathak, G. N., 404

Pauley, S., 386
Payne, J. H., 278, 280, 281, 286, 287, 291, 292
Payne, P., 88
Peake, R. W., 385
Pearsall, W. H., 309
Pease, D. C., 239
Pechmann, E., 443
Peck, R. L., 86
Peel, E. W., 86
Peirce, G. J., 240, 242, 251, 252
Peiris, J. W. L., 182, 184
Pelece, E., 169, 170
Pemberton, C. E., 278
Pendleton, R. C., 388
Perry, T. O., 386
Pertz, D. F. M., 259
Peterson, D. H., 90, 92
Peterson, E. A., 415
Peterson, E. W., 436, 438
Petrie, A. H. K., 18
Pfeffer, W., 237, 241, 247, 249, 252, 253
Phillips, E., 375
Phillips, J. N., Jr., 310, 311, 315, 316, 317, 320, 321
Phillips, W. R., 223
Phillis, E., 370
Phinney, B. O., 194, 195, 198, 201, 204, 206, 207, 209, 398, 399, 400
Piez, K. A., 92
Pilet, P. E., 156, 161, 163, 164, 166, 167, 171, 176
Pinkerton, R. C., 388
Pintner, I. J., 311
Pirie, N. W., 103, 329
Piringer, A. A., 397
Pirson, A., 138, 145, 309, 310, 316, 322
Pisek, A., 258, 266
Pohl, R., 153, 159, 335
Pollard, J. K., 65-114; 65, 66, 67, 68, 70, 71, 72, 74, 75, 76, 77, 78, 80, 82, 83, 84, 86, 87, 88, 89, 90, 92, 104
Pollock, B. M., 164, 167, 173
Pope, D. T., 400, 401
Porter, J. W., 407
Posnette, A. F., 217
Pound, G. S., 415, 416
Powers, W. H., 429
Pramer, D., 335, 413, 415, 418, 419, 421
Pratt, O. E., 281
Pratt, R., 145, 315, 319
Prell, H., 335, 336
Prescott, D. M., 340, 344
Prescott, G. H., 309, 322
Presley, H., 51
Preston, A. P., 218, 226, 228, 229
Preston, C., 104
Preston, R. D., 231, 240, 242, 243, 245, 247, 249, 252, 353
Preuss, L., 310, 321
Prianishnikov, D. N., 66
Priestley, J. H., 242, 244, 246, 249, 250, 252
Prigogine, I., 240
Pringle, R. B., 417
Pringsheim, E., 267
Pringsheim, E. G., 418
Pringsheim, O., 418
Prohorov, N. K., 227
Provasoli, L., 311, 418
Pucher, G. W., 65, 76
Puri, A. N., 278
Purse, D. G., 278

Q

Quackenbush, F. W., 407
Quastel, J. H., 158, 159

R

Raalte, M. H. van, 158, 160, 163, 168
Rabinowitch, E., 120, 124, 125, 126, 128, 129
Rabinowitch, E. I., 115, 121, 122, 125, 137, 138, 145, 146, 147, 148
Rabson, R., 73, 76
Racusen, D. W., 74, 142, 163
Radhakrishnan, A. N., 93
Radley, M., 184, 185, 186, 187, 188, 189, 190, 194, 195, 197, 198, 199, 200, 201, 205, 206, 207, 208, 209, 210, 421
Radner, B. S., 419
Raheja, P. C., 290, 291
Rama Rao, M. V., 300
Ramiah, K., 399
Ramiah, P. V., 299
Randhawa, G. S., 415
Randolph, L. F., 408
Rao, M. S. S., 31
Rao, M. V. R., see Rama Rao, M. V.
Raper, K. B., 186, 187, 188, 189
Rappaport, B. N., 169, 170
Ratner, S., 86, 87, 90
Raub, A., 433
Rautanen, N., 105
Rawlins, T. E., 227
Ray, P. M., 446, 447
Ready, D., 164
Redfield, A. C., 310, 320, 322
Rediske, J. H., 59
Reed, G. B., 102, 104, 105
Rege, R. D., 285, 286
Regeimbal, L. O., 261
Rehm, S., 257
Reid, M. R., 138
Reifer, I., 85, 88
Reimer, F. C., 227
Reineke, L. M., 90, 92
Reinert, J., 156
Reinhold, L., 157
Reiss, E., 162, 164, 167, 171, 172
Renner, O., 237, 239, 244, 247
Rennerfelt, J., 310, 318
Rennert, J., 124
Renwick, M. E., 220, 230, 231
Resch, A., 355
Resende, F., 379
Reusser, P., 415
Reuter, G., 89
Reyes, G. M., 182
Rhoades, M. M., 137
Rhoads, W. A., 49
Richards, B. L., 227
Richards, F. J., 75
Richter, D., 436, 437
Rick, C. M., 397, 398, 407
Ride, M., 415
Rideal, E., 337
Rieman, G. H., 401
Rieth, A., 316
Rigler, F. H., 388
Riker, A. J., 243, 253, 415
Rimington, C., 90, 404
Ripley, T. W., 353
Roach, W. A., 218
Robbins, W. J., 418, 419
Robbins, W. R., 47, 58, 76
Roberts, E. H., 159, 169, 170
Roberts, E. J., 280
Roberts, R. B., 66, 96, 102, 104, 105, 340
Roberts, R. H., 217, 220, 229
Robertson, D. S., 399
Robertson, R. N., 11-30; 11 13, 37, 76
Robin, Y., 90
Robinson, B. A., 396, 398
Robinson, M. E., 436
Robinson, K. C., 421
Robinson, P., 300
Roche, J., 86
Rochovansky, O., 86, 90
Roecki, B., 355
Roewer, F., 443
Rogers, W. S., 217-36; 217, 218, 220, 221, 222, 223, 230
Roller, E. M., 44, 52, 56
Romney, E. M., 49
Ropp, R. S. de, 414, 418, 420
Rosen, W. G., 415, 418
Rosenberg, J. L., 115-36; 130, 133
Rosenberg, T., 371
Rosene, H. F., 335
Rosenfeld, A. H., 301
Rosenthal, T. B., 35
Rostenbach, R. E., 389
Roth, S. N., see Naef-Roth, S.
Rothbur, L., 145
Rothstein, A., 335, 340, 371
Rothstein, M., 75
Rottenberg, W., 269
Rouillard, G., 283

Rouschal, E., 271, 350, 351, 353, 370
Rouse, R. D., 53
Roux, E., 92
Roux, L., 415
Row, K. K., 299
Roza, J. de la, 278
Rozee, K. R., 341
Rubins, E. J., 52
Ruck, H. C., 229
Rufelt, H., 176
Ruhland, W., 335
Rune, O., 382
Russell, C. S., 58, 74, 92
Rutter, A. J., 385
Ryan, F. J., 166
Ryan, V. A., 117, 118
Ryder, A., 72
Rydon, H. N., 67, 86
Ryther, J. H., 387

S

Sabinina, M., 263
Sachs, R. M., 379
Sadaoka, G., 286, 287, 291, 292
Sadasivan, T. S., 187
Saito, T., 283, 285, 286
Salisbury, F. B., 382
Salmon, J., 354
Salton, M. R. J., 344
Salzman, N. P., 72
Samuel, E. W., 335
Samuel, G., 360
Samuels, E. W., 230
Samuels, G., 34, 282, 283, 299, 300, 301
Sancier, K. M., 116, 124
Sandegren, E., 66
Sannabhadti, S. K., 285, 286
Santamaria, P. R., 239
Sanyal, P. B., 301
Saris, N., 97
Sarojini, T. S., 187
Sartoris, G. B., 292, 293, 301
Sasa, T., 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 322, 323, 325, 327
Sass, J. E., 406
Sauthoff, W., 415
Sawada, K., 182, 183, 184, 194, 195, 197, 200
Sax, K., 221, 225, 226
Saxena, J. S., 280
Saz, A. K., 97
Scarisbrick, R., 137, 144, 147
Scarth, G. W., 264, 271
Schaeffer, P., 418
Schaffner, G., 417
Schaffner, J. H., 395
Schatz, A., 311, 418
Schenck, G. O., 122
Schermerhorn, L. G., 47
Schilling, E. D., 73
Schimper, A. F. W., 257
Schindler, H., 343
Schlessinger, M. J., Jr., 38, 40
Schmadlak, J., 227
Schmidt, E., 243, 253
Schmidt, H., 257, 271, 272
Schmitt, F. O., 2
Schneider, G. W., 261, 263, 264, 266
Scholander, P. F., 238, 239, 240, 242, 244, 247, 248, 249, 253
Schön, W. J., 316
Schopfer, W. H., 169, 415, 418, 419
Schou, L., 74, 89
Schov, L., 444
Schramm, G., 103
Schrock, O., 408
Schroo, H., 285, 287
Schubert, W. J., 278
Schultz, J. H., 227
Schulz, F., 221, 227
Schumacher, W., 349, 350, 351, 353, 354, 356, 357, 368, 370
Schwan, H. P., 340
Schwantes, H. O., 342
Schwartig, A. E., 398, 399
Schwartz, M., 145
Schweet, R. S., 74, 90
Scofield, H. T., 376
Scott, A. F., 239
Scott, G. T., 34, 53, 310, 320
Scott, L. I., 244
Scott, R., 55
Searle, V. H. L., 238
Seebeck, E., 72
Seely, R. C., 159
Segelitz, G., 161, 168, 169
Seiler, L., 156, 164, 166, 167
Sell, H. M., 201, 206
Selman, F. L., 53
Seltmann, H., 398
Sen, P. K., 104
Sen, S. C., 290
Serr, E. F., 229
Seta, Y., 186, 187, 188, 189, 190, 191, 192, 193, 194, 204, 207, 210
Seto, F., 182, 183, 184, 185, 186, 198, 207
Sexton, W. A., 176
Shachori, A. Y., 380
Shafer, J., 397, 399
Shah, J. D., 83
Shamel, A. D., 277, 279
Sham Singh, 221, 223, 226
Shannon, L. M., 49
Shantz, E. M., 85
Shapiro, D., 72
Shaw, B., 408
Shaw, H. R., 297, 301
Shaw, J. K., 224
Shaw, M., 264
Shear, C. B., 48
Shedlovsky, T., 2
Shekleton, M. C., 408
Sheldon, J. L., 183, 210
Shemin, D., 58, 74, 92
Shibata, K., 146, 310, 311, 312, 313, 314, 315, 316, 318, 320
Shigeura, G., 295
Shimada, S., 184, 186, 187, 194, 198, 199, 206, 207
Shipton, M. E., 171
Shive, W., 73
Shoemaker, D. P., 239
Short, W. A., 138
Shug, A. L., 52
Shull, C. A., 237, 238, 250
Sideris, C. P., 49, 57, 143
Siddiqi, A. M., 444, 445
Siegel, S. M., 54, 162
Sievers, J. F., 20
Signol, M., 415, 418
Silva, J. A., 286, 287, 291, 292
Silver, R. S., 240
Silver, W., 95
Simmonds, G. H., 239
Simon, E. W., 341, 440
Sinclair, W. B., 68
Singer, T. P., 428
Singh, B., 404
Singh, B. N., 288
Singh, K. K., see Kirpal Singh, K.
Singh, L. B., 229
Singh, M. P., 288
Singh, R. K., 183
Singh, S., see Sham Singh
Singleton, W. R., 395, 396
Sisakian, N. M., 261
Sisler, E. C., 41, 51
Siu, R. G. H., 183, 186
Sjörs, H., 387
Skaggs, D. P., 204, 207
Skelding, A. D., 17, 22
Skinner, F. A., 413
Skipper, H. E., 138
Skok, J., 47
Skolko, A. J., 415
Skoog, F., 55, 158
Skwarra, H., 156
Slagg, C. M., 184, 210
Slankis, V., 168, 170, 173
Slattery, M. C., 263, 272
Slatyer, R. O., 381
Slie, R. B., 97
Sloane, G. E., 286, 287, 291, 292
Slonimski, P. P., 341
Small, J., 143
Smit, A. J. H., see Haagen-Smit, A.
Smith, B. A., 280
Smith, D., 386
Smith, D. E., 203, 206
Smith, D. G., 68, 79
Smith, F., 237, 238, 250
Smith, F. G., 436, 445

Smith, G. M., 240, 243, 245, 250
Smith, I., 79
Smith, J. H. C., 116, 125, 146, 310, 313, 314, 316, 317, 318, 319, 320, 321, 398
Smith, P. G., 399, 408
Smith, P. W. G., 67, 86
Smith, R. L., 34
Smith, R. M., 298
Smith, W. W., 222
Snow, G. A., 428
Snow, R., 154, 162, 171
Snyder, E., 229
Snyder, W. C., 183
Soboleva, O. I., 267, 269
Sobr, M.O.C.B., see Brasil Sobr, M.O.C.
Söding, H., 160, 162
Sohns, V. E., 186, 187, 188, 189
Somers, G. F., 377
Sørensen, T., 376, 386
Sorokin, C., 319
Sotty, L., 420
Sotty, O., 418
Southwick, L., 224
Specht, A. W., 37, 44, 52, 56, 58
Spector, L., 87
Spence, D. J., 191
Spencer, D., 31, 44, 45, 93
Spiegelman, S., 100
Spikes, J. D., 115, 120, 131, 137, 141, 142, 144, 145, 146, 147, 148, 149
Spoehr, H. A., 261, 275, 309, 310, 313, 314, 316, 317, 318, 319, 320, 321, 329, 397
Sporer, A. H., 116
Sprague, G. F., 406, 409
Sprinson, D. B., 74
Srb, A. M., 86, 90, 393
Sreerangachar, H. B., 439
Srinivasan, P. R., 74
Srivastava, D. P., 301
Srivastava, J. S., 290, 301
Srivastava, S. P., 280
Staab-Müller, R., 158, 159
Stafford, H. A., 74, 427
Stahmann, M. A., 413, 415, 416
Stålfelt, M. C., 258, 263, 271
Stanley, R. G., 380
Stanley, W. M., 6
Starkey, R. L., 415, 420
Stebbins, M. E., 418, 419
Steers, F., 88
Stein, W. H., 68, 69
Steinberg, R. A., 44, 51, 52, 56, 75, 396, 434
Steinberger, A. L., 258
Steindl, D. R. L., 276
Steinman, H., 429
Stender, H. K., 295
Stenlid, G., 439
Stephens, R., 42
Stephenson, M. L., 100, 106
Stepka, W., 67, 74, 89
Stern, H., 77
Steuerwald, L. G. L., 278
Stevens, G. de, 278
Stevens, P. G., 12
Stevens, R., 281
Steward, F. C., 65-114; 34, 35, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 92, 93, 97, 98, 101, 102, 103, 104, 105
Steyn, D. G., 210, 404
Stigter, H. C. M. de, 224
Stiles, W., 17, 22
Stocker, O., 257, 264, 272
Stocking, C. R., 137, 141, 237, 238, 243, 244, 245, 253, 267, 427
Stodola, F. H., 186, 187, 188, 189, 191, 192
Stokes, A., 415, 422
Stokes, G. W., 408
Stoll, A., 72, 141, 142, 144, 146
Stoll, C., 184, 188, 206, 207, 210
Stone, E. C., 380
Stotz, E., 436, 445
Stout, M., 144
Stout, P. R., 44, 52, 53, 57, 104, 361, 381, 383
Stowe, B. B., 181-216; 74, 155
Strain, H. H., 310, 317, 319
Strasburger, E., 237, 249
Stratmann, H., 317, 318, 319, 320, 321, 322
Street, H. E., 34, 35, 65, 66, 97, 102, 104, 159, 169, 170
Strehler, B. L., 128, 129
Strmecki, E. L., 58
Strong, F. M., 73
Struckmeyer, B. E., 253
Strugger, S., 237, 238, 258, 352, 354
Stubbs, J., 413, 415
Stubbs, R. W., 39, 48
Stubbs, W. C., 278
Stumpf, P. K., 42, 43, 49, 56, 94, 445
Stupp, R., 121
Subramanian, D., 417
Sullivan, J. T., 401
Sumiki, Y., 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 197, 198, 199, 200, 201, 204, 205, 206, 207, 208, 210
Sumner, J. B., 444
Sumner, R. J., 444
Sun, C. N., 172
Sundman, J., 74
Suomalainen, H., 342
Sussex, I. M., 159, 170
Sutcliffe, J. F., 17, 22
Sutherland, G. L., 73
Sutter, E., 157
Sutton, M. D., 415
Suzuki, K., 200, 201, 204, 206, 283, 284, 285, 286, 316
Suzuki, Y., 437
Svedberg, T., 140
Swanson, C. A., 363
Swanson, C. P., 164
Swarbrick, T., 220, 221
Swezey, J. A., 298
Sylven, H., 378, 386
Synge, R. L. M., 72
Syre, H., 162, 167, 176
Szent-Györgyi, A., 429, 431, 433

T

Tadokoro, T., 428
Tadros, T. M., 381, 382
Tager, J. M., 105
Taggart, J. V., 371
Takada, H., 53
Takahashi, D., 275-308; 283, 287
Takahashi, N., 186, 187, 188, 189, 190, 191, 192, 193, 194, 204, 207, 210
Takahashi, T., 182, 184, 186, 197, 198, 200, 201, 207
Takahashi, W. N., 103
Takai, M., 186, 187, 188, 189, 190, 191, 192, 193, 194, 204, 207, 210
Takashima, S., 130, 133
Takasugi, N., 428
Takeda, Y., 42, 49
Takeshita, T., 293
Takijima, Y., 194, 197, 199, 200, 201, 203, 207
Takimoto, S., 181
Talbot, B. E., 67
Tamari, K., 90, 188, 189, 191, 192, 199, 200, 207, 208
Tamiya, H., 309-34; 146, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 328
Tamiya, N., 328
Tamura, S., 186, 187, 188, 189, 190, 191, 192, 193, 194, 204, 207, 210
Tamura, T., 188, 189, 191, 192, 199, 207
Tanada, T., 33, 35, 46
Tanenbaum, S. W., 74
Tang, Y. W., 163, 446
Tanimoto, T., 275-308; 283, 284, 286, 287, 291, 292
Tanner, H. A., 147, 148
Tanner, K. N., 239

XUM

Tanret, M. C., 406
Tansley, A. G., 387
Tappel, A. L., 444, 445
Tarpley, W. B., 428, 429, 435, 436, 437
Tarver, H., 100
Tatchell, A. R., 67, 68, 81
Tatum, E. L., 74, 403
Taubock, K., 85
Tauja-Thielman, M. J., 169, 170
Taylor, F., 159
Taylor, W. R., 72
Tazawa, M., 339
Teas, A. N., 404
Teas, H. J., 393-412; 401, 402, 404, 408
Tempany, H. A., 297
Temperely, H. N. V., 239, 241
Tendeloo, H. J. C., 20
Terner, C., 27
Terrell, A. J., 439
Terui, M., 186, 187
Thaine, R., 362
Theorell, H., 443
Thiel, K., 225
Thielman, M. J. T., see Tauja-Thielman, M. J.
Thimann, K. V., 49, 55, 74, 100, 106, 154, 155, 158, 159, 162, 164, 166, 175, 335, 419, 428, 432, 446, 447
Thoai, N. van, 86
Thoday, D., 264
Thomas, C. A., Jr., 34
Thomas, H. E., 227
Thomas, J. B., 138, 140, 141, 143, 144, 146
Thomas, K. M., 182, 183
Thomas, L. A., 221
Thomas, W. H., 56, 311
Thompson, E. C., 218, 230, 231
Thompson, G. P., 280, 281
Thompson, J. F., 65, 66, 69, 70, 71, 72, 73, 75, 76, 77, 78, 81, 82, 93, 97, 103, 105
Thorne, D. W., 58
Thresh, R., 155, 157, 161, 171
Thut, H. F., 244
Tiffany, L. H., 309, 322
Tikkanen, R., 339
Timonin, M. I., 415
Tissières, A., 441
Tochinai, Y., 201, 207
Toivonen, N. J., 66
Tolbert, N. E., 39, 40, 46, 74, 84, 89, 142, 444
Tomes, M. L., 407
Tomita, T., 86
Tong, W., 54
Toole, E. H., 375, 376, 378, 395, 397, 399
Toole, V. K., 375, 376, 378, 395, 397, 399
Torii, H., 197, 200, 201, 207
Torrey, J. G., 174, 175
Toschi, G., 73
Totter, J. R., 42
Towers, G. H. N., 65, 72, 73, 74, 79, 81, 82
Tracey, M. V., 85, 86, 88
Traub, H. P., 263, 272
Trelease, S. F., 46
Troll, C., 379
Troll, H. J., 408
Tso, P. O. P., 106
Tubbs, F. R., 217
Tukey, H. B., 38, 39, 87, 208, 217, 221
Tukey, R. B., 222
Tung, Y. T., 258
Tupicyn, D. I., 220
Turesson, G., 386
Turkina, M. V., 364, 365, 366, 367, 368
Turner, J. S., 29
Tydeman, H. M., 226, 229

U

Uhlig, H. H., 2
Uksila, E., 72
Ullstrup, A. J., 184, 185
Ulrich, A., 53, 54, 55
Umbarger, H. E., 74
Umrath, K., 345
Umrikhima, A. V., 123
Uno, S., 186, 187, 207
Unwin, C. H., 415, 416, 417
Urbach, G., 90
Urey, H. C., 66
Uri, N., 123, 132, 147
Url, W., 143, 339, 342
Ursprung, A., 237, 239, 244
Ussing, H. H., 344

V

Vaartaja, O., 386
Vaidyanathan, C. S., 86
Valleau, W. D., 408
van den Honert, T. H., see Honert, T. H. van den
Van der Veen, R., 147, 148
van Dillewijn, C., see Dillewijn, C. van
Vanecko, S., 45
Van Fleet, D. S., 350
Van Niel, C. B., 137, 138
van Oorschot, J. L. P., see Oorschot, J. L. P. van
van Overbeek, J., see Overbeek, J. van
van Raalte, M. H., see Raalte, M. H. van
van Wagtendonk, W. J., see Wagtendonk, W. J. van
Varahalu, T., 299
Varner, J. E., 45, 88
Vatter, A. E., 116
Vaughn, J. R., 415
Venkataraman, T. S., 299
Venkatesan, T. R., 85
Vennesland, B., 74, 433
Verduin, J., 263, 387, 388
Vergnano, O., 59
Vernon, L. P., 362, 363
Verret, J. A., 296
Verschaffelt, E., 85
Vervelde, G. J., 20
Vickery, H. B., 65, 76
Vieregg, A., 169, 173
Vieweg, G. H., 264
Vijayasaradhy, N., 292, 293
Vince, D., 204
Vincent, R. S., 239
Vines, S. H., 237, 243
Virgin, H. I., 125, 339
Virtanen, A. I., 65, 68, 71, 72 74, 82, 84, 87, 90, 92, 93, 97
Vishniac, W., 94, 115, 137
Vittorio, P. V., 104
Vogel, H. J., 74, 90
Volcani, T. E., see Elazari-Volcani, T.
Volkmann, C. M., 437
Volz, G., 352
von Abrams, G. J., see Abrams, G. J. von
von Denffer, D., see Denffer, D. von
von Euler, H., see Euler, H. von
Von Hackbarth, J., 403
Von Schwarze, P., 404
Von Sengbusch, R., 403
von Witsch, H., see Witsch, H. von
Voorhees, R. K., 183, 184, 185
Voorspuij, A. J. Z., see Zwart Voorspuij, A. J.
Vyvyan, M. C., 218, 220, 221, 230

W

Wada, B., 195, 207
Wada, M., 87
Waddell, C. W., 301
Wadleigh, C. H., 48, 58, 261
Wadsworth, H. A., 297, 298
Wagenknecht, A. C., 42, 50, 163, 446, 447
Waggoner, P. E., 413
Wagner, R. P., 74, 393, 394
Wagtendonk, W. J. van, 280
Wailes, P., 80, 81
Wain, R. L., 159
Wakeman, A. J., 65, 76
Walker, H. M., 47, 57, 381
Walker, J. B., 44, 48, 52, 87, 88
Walker, J. C., 401, 413
Walker, R. B., 47, 57, 381, 382
Walker, T. A., 74

Wallace, A., 34, 40, 49, 56, 229
Wallace, R. H., 398, 399
Wallace, T., 223
Walldov, E. E., 141, 143, 144, 145
Wallen, V. R., 415
Wallihan, E. F., 50, 58
Walsh, K. A., 444
Walter, H., 267, 271, 385
Walters, M. C., 362
Walton, B., 102
Wang, F. H., 415
Wanner, H., 354, 355
Warburg, O., 138
Ward, G., 189
Wareham, R. T., 376
Wareing, P. F., 378
Waring, E. J., 44
Warington, K., 57
Waris, H., 342
Warmke, G. L., 158
Warmke, H. E., 73, 97, 158
Warne, L. G. G., 245
Wartiovaara, V., 339
Warwick, A. J., 90, 92
Wasicky, R., 278
Wassink, E. C., 197, 198, 202, 206, 310, 311, 313, 314, 317, 322, 379, 386
Watanabe, A., 45, 324, 325, 327
Watt, A. S., 385
Way, A. M., 413, 415
Waygood, E. R., 431
Wayrynen, R. E., 146, 148
Wear, J. I., 50, 57
Webb, J. A., 65, 74, 82
Webb, J. L., 84
Webber, H. J., 229
Weber, F., 258
Webster, G. C., 66, 88, 93, 98, 99, 100
Wedding, R. T., 68, 341
Weier, T. E., 137, 138, 427
Weigl, J. W., 116, 124, 310
Weinstein, L. H., 58
Weintraub, R. L., 446
Weiss, J., 124
Weiss, M. G., 396, 400, 408
Weiss, U., 74
Weissbach, A., 105
Weissman, G. S., 46
Weller, A., 124
Weller, D. M., 297
Weller, L. F., 201, 206
Wendel, J. G., 239
Went, F., 159
Went, F. W., 139, 155, 167, 205, 208, 375, 407, 447
Werle, E., 443
Wessel, G., 52, 54, 310
Wessels, J. S. C., 147, 148
West, C., 205, 223
West, C. A., 194, 204, 400
West, D. W., 402
Westall, R. G., 75
Westergaard, W., 375
Wet, A. F. de, 219, 220
Wetmore, R. H., 70, 71, 74, 76, 77, 82
Whaley, W. G., 396
Whalley, B. E., 85
Whatley, F. R., 54, 58, 142, 148
Wheeler, H. E., 409
Whistler, R. L., 406
Whitaker, T. W., 398, 408
White, F. C., 86
White, O. E., 408
White, W. L., 183, 186
Whiteley, A. H., 239
Whiting, G. C., 439
Whiting, M. C., 80, 81
Whitman, W. C., 272
Whitney, I. B., 42
Whitney, J. B., Jr., 362, 363
Whittaker, R. H., 381, 382, 387
Whittingham, C. P., 137, 138
Whyte, D. E., 280
Wickson, M. E., 74, 79, 81
Wiebe, H. H., 38, 39, 40, 46
Wiersma, J. H., 379, 386
Wiggins, L. F., 278, 279, 280, 281
Wightman, F., 159
Wilbrandt, W., 371
Wildman, S. G., 66, 105, 139, 140, 402, 437, 439
Wilkins, M. J., 37
Wilkinson, B. R., 227
Wilkinson, S., 90, 92
Williams, A. H., 230
Williams, J. H., 280, 281, 310, 321
Williams, R. C., 103, 344
Williams, R. F., 38
Williams, W. T., 258
Willstatter, R., 141, 142, 144, 146
Wilner, J., 228
Wilson, C., 257
Wilson, D. B., 385
Wilson, D. G., 84, 105
Wilson, E. B., 1
Wilson, J. W., 379
Wilson, P. W., 52, 93, 95
Wilson, R. A., 415, 418
Wilson, R. D., 44
Winkler, E., 258, 266
Wintermans, J. F. G. M., 316
Wismer, K. L., 239
Witkop, B., 92, 93
Witsch, H. von, 309, 310, 319, 320, 321, 322
Witt, H. T., 130
Wittwer, S. H., 38, 39, 87, 200, 201, 202, 203, 205, 206, 207
Wolcott, L., 310, 321
Wolf, H. L., 92
Wolfe, J. N., 376
Wolfe, M., 94
Wolffgang, H., 89
Wolfrom, M. L., 278, 280, 281
Wollenweber, H. W., 182
Wood, B. J., 407
Wood, J. C., 72
Wood, J. G., 66, 93, 97, 261, 272
Wood, R. K. S., 183
Wood, W. A., 74
Woodhams, D. H., 261, 263
Woodhouse, E. D., 244, 249
Woodstock, L., 316
Woodworth, C. M., 199, 210
Woolley, D. W., 416, 417
Woolley, J. T., 52, 310
Wosilait, W. D., 439
Wotherspoon, N., 124
Wright, E. C. B., see Barton-Wright, E. C.
Wright, J. G., 243
Wright, J. M., 413, 415, 416, 417, 418, 419
Wright, K. E., 37
Wright, N., 70, 74, 92
Wriston, J. C., Jr., 58
Wulzen, R., 280
Würgler, W., 168, 169
Wuttke, H., 404, 409
Wyatt, K. S., 239
Wyssling, A. F., see Frey-Wyssling, A.

Y

Yabuta, T., 186, 187, 188, 189, 191, 192, 194, 197, 198, 199, 200, 201, 202, 204, 205, 206, 207, 208, 210, 421
Yamada, T., 418
Yamagutchi, S., 438
Yamaki, T., 181-216
Yamamoto, Y., 435
Yamasaki, M., 288, 299
Yamauti, K., 183, 184
Yap, G. G., 288
Yasue, Y., 416
Yatazawa, M., 189
Yearwood, R. D. E., 278
Yemm, E. W., 68, 69, 84, 101, 104
Yin, H. C., 258
Yocum, C. S., 138, 428, 432, 443
Yoder, P. A., 280
Yogeswari, L., 187
Yoshida, R. K., 73
Yoshii, H., 185, 195, 198, 207
Yoshimura, T., 292
Young, E. G., 68, 79
Young, H. Y., 49, 57, 143
Young, L. C. T., 433
Young, T. F., 238

XUM

Ysselstein, M. W. H. van, 157, 163
Yuen, Q. H., 282

Z

Zabin, I., 407
Zachariah, A. T., 163
Zacharius, R. M., 66, 67, 68, 70, 71, 72, 75, 78, 80, 81, 86, 87, 89, 90
Zahn, F. P., 227
Zähner, H., 188
Zahner, R., 381
Zalensky, V., 269
Zamecnik, P. C., 100, 106
Zaprometov, M N., 368, 369
Zech, H., 359
Zechmeister, L., 407
Zeiger, D. C., 227, 228
Zelitch, I., 444
Zeller, E. A., 443
Zerban, F. W., 278, 280
Zerban, K., 430
Zernova, L. K., 267
Zeuthen, E., 344
Zidan, Z. I., 229
Zill, L. P., 142
Zilva, S. S., 428
Zimmerman, M., 361
Zucker, M., 44
Zwart Voorspuij, A. J., 20
Zweig, G., 67, 69

SUBJECT INDEX

A

Acid
  as inhibitor of Hill reaction, 143
  organic, see Organic acid
  permeability of, 341-42
  and root geotropism, 176
  see also Gibberellins
Aconitase
  metal and, 42
Actidione
  see Antibiotics
Actinic light, 129
Active transport
  see Uptake
Adenosinetriphosphate, as absorption carrier, 33
Adenylic kinase, metals and, 42
Adsorption, exchange, 32-34
Agar, auxin collection in, 154, 158
Age, and graft incompatibility, 225
Agriculture, and gibberellins, 209-10
Alanine, 67
Albinism, 418
Algae
  amino acids and, 45
  calcium and, 46, 48
  chloroplasts in, 138
  culture of, 309-29
  dessication and, 269
  Hill reaction in, 145
  marine, 68
  metal chelates and, 49
  nitrogen-fixing, 324-25
  red, 1
  in sewage, 325-27
  yield in culture, 321-24
Alkaloids, gene for production, 403-4
Allantoic acid, 88-89
Allantoin, 88-89
Alternaric acid, 417
Aluminum, as enzyme activator, 54
Amides, 81
Amine oxidase, 443
Amino acid
  of algae, 45
  animal nutrition and, 73-75
  chromatography and, 69-80
  cyclic, 91
  growth and, 76-78
  keto acids and, 71-74
  occurrence of, 70-73
  primitive formation of, 66
  in sugarcane, 280
  translocation of, 368
  uptake, 335
Ammonia
  role of, 84-85, 87, 93
  in sugarcane, 287
Angiosperms, sieve area in, 353, 355
Anilides, 85
"Anion respiration," 36
Antagonism, ion absorption and, 35
Anther, fluorescence in, 402
Anthocyanin, antibiotics and, 419
Antiauxins, 173-76
Antibiotics, 145
  bacteria and, 413
  effect on plants, 413-23
  fungus and, 413
  growth and, 419-21
  as inhibitors, 414-19
  nomenclature, 414, 415
  pigments and, 418-19
  production in soil, 413
  translocation of, 421-22
Apparent free space, 11-29, 32, 337
  definitions, 12-14
  determination of, 14-20
  external solution and, 26-27
  properties of, 20-29
  significance of, 28-29
  sugar and, 24
  volume of, 23-25
Apple, stock and scion relations, 217, 223
Aquatic plants, 1-9
Arctic-alpine species, 379
Arginine
  accumulation of, 89-90
  chromatography and, 69
  detection of, 86
  sulfur deficiency and, 75
  urea from, 85
Ascorbic acid
  and gibberellins, 201
  and iron, 49
  as photobleach, 123
  rootstock effect, 223
Ascorbic acid oxidase, 428-34
Asparagine
  chromatography and, 68, 76
  inhibited translocation of, 365
  in sugarcane, 280
  translocation of, 368
  see also Nitrogen metabolism
Aureomycin, see Antibiotics
Auxin
  and algal growth, 315
  antiauxins, 173-76
  binding of, 162-63
  biochemistry of formation, 158
  cobalt as, 55
  destruction of, 163-64
  external and root growth, 164-73
  external factors and production of, 160-61
  "free," 156
  genes and, 399
  geotropism and, 176
  glucose and, 160
  in grafts, 231-32
  growth regulators and, 161
  identification of, 155
  inhibitory nature, 172-73
  and intact roots, 164-68
  and isolated roots, 168-71
  light and, 161
  nitrogen and, 160-61
  occurrence in root, 154-58
  oxygen and, 160
  phosphate and, 160
  phototropism and, 176-77
  production in root tips, 158-60
  protein and, 162
  relations in roots, 153-77
  respiration and, 160
  and root sections, 171-72
  and root swelling, 172-73
  in sugarcane, 281
  transport in roots, 161-62
  treatment after-effects, 166
  uptake, 157
  see also Gibberellins
Azetidine-2-carboxylic acid, 93
Azo dyes, reduction of, 122

B

Bacillomycin A, see Antibiotics
Bacteria
  and algae, 325
  permeability of, 340
  photosynthetic, 138
  protein and amino acid metabolism in, 66
Bagasse, 277-78
Bakanae, 181
Banana, chromatographic studies, 76
Barium, absorption of, 53
Barley
  fungus, 183
  and gibberellins, 198

Bases, permeability of, 341-42
Beans, fungus of, 189
Biolectric studies, 35
Blooming, day length and, 379
Blossom, rot of, 46
Boron
  calcium and, 56
  deficiency of, 51, 400
  nicotine synthesis and, 51
  and rice fungus, 187
  sugar and, 41, 51
  transpiration and, 51
Bract, germination and, 376
Bromine, absorption of, 53
Bud
  calcium in, 38
  drought and, 271
  and sap ascent, 250

C

Calcium
  as absorption carrier, 33
  absorption of, 34
  in algae, 46, 48
  boron and, 56
  nitrogen metabolism and, 47
  physiological effect of, 46-48
  and plant distribution, 382
  and plasmalemma formation, 345
  potassium and, 56
  respiration and, 36
  sodium and, 56
  sulfur movement and, 46
  translocation of, 38-39
Callose, 352-54, 365
Cambium, 246
Capillarity, 238
Carbamyl compounds, 84-90
Carbohydrate
  absorption of, 291
  genetics of storage, 406
  and gibberellins, 200
  metabolism in green tissues, 261-62
  transfer in phloem, 354
  translocation of, 291
Carbon dioxide
  and algal culture, 309, 310
  assimilation, 140, 142
  root absorption and, 31-32
  stomata and, 258
Carbon tracers, 37
Carbowax, and chloroplast, 141
Carbowax-cytoplasm, 145
Carotenoids, 125
  antibiotics and, 419
  genetic factor for, 407-8
Carriers, 6-7
  root absorption and, 32-35
Catalase, in chloroplasts, 140
  copper and, 42
Cell
  absorption spectra, 125
  acids in, 341-42
  in algal growth, 315
  apparent free space, 11-29
  artificial, 7
  bases in, 341-42
  companion, 355-56
  division, 195
  dye permeation of, 342-44
  electrical properties of, 8
  electrolytes in, see Ions
  elongation, 174-75, 194
  green, phosphorescence in, 128
  injury of, 3
  osmotic pressure and, 268
  permeability of, 335-46
  plasma membranes, 344-45
  protein metabolism of, 101-3
  resistance to dessication, 269-72
  salts and ions in, 340-41
  sap and permeability, 343
  sap, composition of, 4, 5
  sites of resistance to penetration, 336-38
  sugar in, 11
  thermal deterioration of, 145-46
  wall, see Cell wall
  in water transport, 249-50, 251
  see also Permeability
  see also Uptake
Cell wall
  apparent free space and, 24
  and ascorbic acid oxidase, 432
  calcium and, 48
  osmosis and, 336-37
Cellulose, gibberellin fungus and, 186
Cesium, absorption of, 53
Chelates, in algal culture, 311
Chloramphenicol, see Antibiotics
Chlorella
  Hill reaction in, 145
  inhibitor of, 417
  molybdenum and, 52
  spectroscopy, 125, 126, 127
  urea and, 88
  see also Algae
Chloride, as germination inhibitor, 376
Chlorine
  absorption of, 53
  deficiency of, 54
Chloroform extraction of auxin, 154
Chloromycetin, see Antibiotics
Chlorophyll
  antibiotics and, 418
  biosynthesis of, 124-25
  bleaching of, 118
  crystalline, 116
  excited, 121
  fluorescence and, 120-21, 125-31, 133-34
  genetic deficiency of, 397-99
  and gibberellins, 199
  in vitro, 115-24
  infrared spectrum, 116
  phosphoresence, 119
  photobleaching of, 112, 123
  photochemistry of, 115-34
  potassium and, 43
  sensitized reactions of, 121-24
  spectroscopy and, 115-20
  synthesis and photosynthesis, 146
  see also Hill reaction
Chloroplast
  biochemistry of, 137-49
  contraction of, 5
  and gibberellins, 199
  growth, 137
  in Hill reaction, 137-49
  inheritance, 137
  iron in, 40
  isolated, 138-42
  leaf, 125
  ontogenetic factor, 146
  osmotic pressure and, 139
  phospholipids in, 145
  phosphorescence in, 128
  purification of, 138-42
  redox catalysts of, 147-48
  structure, 137, 138
  swelling of, 139
  water movement and, 5
  see also Chlorosis
Chlorosis, 57-59
  antibiotics and, 418
  and gibberellins, 199
  metal chelates and, 49
  in sugarcane, 281
  tracers in, 31
Chlortetracycline, see Antibiotics
Chromatography
  auxin identification and, 155-56
  genetics and, 78-80
  growth and, 76-78
  in nitrogen metabolism studies, 65, 66, 80, 86
  seed and, 76
  taxonomy and, 78-80
  xylem sap and, 79
Citrus, molybdenum deficiency and, 52
Clavacin, see Antibiotics
Clavatin, see Antibiotics
Climate
  and growth, 294-302
  microclimates, 376-77
  and translocation, 291
Clones, see Grafting
Cobalt
  as hormone, 55

XUM

peroxide formation and, 54
as red light, 54
requirements for, 55
translocation of, 40
Coleoptile, growth regulators in, 154
Colloids, drought resistance and, 272
Color, genes for, 397-99
Companion cell, 355-56
Competition, 383-35
Conduction, see Sap ascent
Copper
catalase and, 42
indoleacetic acid and, 42
magnesium and, 56
molybdenum and, 56
photosynthesis and, 50
Corn
auxin in, see Auxin
reproductive processes, 50
Cotton, sodium and, 53
Cress, auxin in root, 156
Cucumber, and gibberellin, 197
Cuticle, 264
Cuttings, 228
Cyanide, enzyme for production, 401
Cyanogenesis, 401
Cycloheximide, see Antibiotics
Cytochrome oxidase, 140, 431, ions and, 36
Cytochromes, 428
Cytoplasm
cations in, 20
as Donnan system, 17-18, 20-24
free space in, 13
osmotic pressure of, 25-26
structure of, 344

D

Dark
absorption in, 37
translocation in, 37
Day length
blooming and, 379
seedling growth and, 378
see also Light
Dehydroporphin, 116
Desmids, 138
Dew, as water source, 380
Diatoms, 336
2,4-dichlorophenoxyacetic acid, 157, 161
Dicots, cation exchange in, 34
Diffusion, facilitated, 336
Dihescence, 408
Diseases resistance, 226-29
genes for, 401
Donnan system, 17-18, 20-24, 34
Dormancy, 375
breaking of, 378
Drought resistance
carbohydrate metabolism and, 261-62
cell resistance to dessication, 269-72
methods of study, 257
osmotic pressure and, 267-69
photosynthesis and, 262-64
physiological processes and, 257-72
respiration and, 264-66
stomatal movement, 258-61
water content variations, 257
Drugs, 145
Dwarf plants, 195-96
Dwarfing
interstocks and, 221
virus and, 217
Dwarfness, genes and, 399-400
Dyes
movement in phloem, 356-58, 362
permeation of, 342-44

E

Ecology
comparative, 385-87
competition, 383-85
ecosystems, 387-89
germination studies, 375-76
microclimates, 376-77
photoperiodism, 377-79
physiological, 375-89
radiation, 388
soil, 381-83
water relations, 379-81
Ecosystem, 387-89
Ecotypic variations, 386
Electrolytes, see Ions
Elements, physiological effects of, 43-55
Energy
metastable state, 127
of photosynthesis, 119-20, 123
in plastid, 127
quantum conversion in photosynthesis, 148-49
transfer in Hill reaction, 146
two-quantum theory, 131-34
Enzymes
activity in conducting tissue, 366
aluminium and, 54
auxin and, 158
auxin-destroying systems, 163
copper and, 50
for cyanide production, 401
in determination of nitrogenous compounds, 86
genes and, 393-94
gibberellins and, 200
grafting and, 224
inhibitors of, 84
manganese and, 50
metals and, 42
molybdenum and, 52
nickel and, 54
in nitrogen metabolism, 66, 93
oxygen liberation and, 132
pectic, 48
in phloem, 354
in rubber production, 402
in sieve elements, 350-51
and stomatal movement, 258
in sugarcane, 290, 291
see also Soluble oxidases
Enzyme systems, ion action on, 36
Epinasty, 196-97
Epidermis, see Stomatal movement
Evapotranspiration, 381
Expansine, see Antibiotics

F

Fat, algae as source of, 327
Fern, 76, 79
Fiber, of sugarcane, 278
Flavonoid pigments, 404-6
Flavoproteins, 442-43
Flax, auxin in, see Auxin
Flowering
amino acids and, 75, 78
cold requirement for, 406
genetic factor for, 406
gibberellins and, 202
light and, 396
of sugarcane, 292-94
Fluorescein, 119, 358, 362
movement of, 363
Fluorescence
of chlorophyll, 120-21
genetic factor, 401-2
lifetime of, 120
steady state, 126-27
water and, 125
Fog, as water source, 380-81
Fragrance, genetic factor for, 407
Fruiting
bridge, 221
effect of interstock, 223
effect of rootstock, 222-23
gibberellins and, 203
Fungocin, see Antibiotics
Fungus
bakanae, see Gibberellins
rice disease, see Gibberellins
Fusaric acid, as inhibitor, 188

G

Gelatin, auxin collection in, 154
Gene
  effect, 393-95
  enzymes and, 393-94
  mutation, 394
  pleiotropic, 394
  see also Genetics
Genetics
  alkaloids and, 403-4
  B-vitamin content and, 408
  carbohydrate storage and, 406
  carotenoids and, 407-8
  chlorophyll deficiencies and, 397-99
  cyanogenesis and, 401
  disease resistance and, 401
  dwarfness and, 399-400
  element utilization and, 400-1
  flavanoids and, 404-6
  flowering and, 406-7
  fluorescence and, 401-2
  fragrance and, 407
  gene effect, 393-95
  germination and, 399
  growth factors and, 402-3
  light response and, 395-97
  lipides and, 404
  nicotine and, 408
  physiological, 393-409
  rubber synthesis and, 402
  survival in competition, 384
Geotropism
  genetics of, 399
  in roots and auxin, 176
Germination, 375-76
  antibiotics as inhibitors of, 414-16
  and gibberellins, 203
  of sugarcane, 296, 298
  urea and, 85
Gibberellins, 181-210
  agricultural, 209-10
  auxin and, 205-8
  bioassay and terminology, 208-9
  biological activity of, 193
  cell division and, 195
  cell elongation and, 194
  characteristics of, 186
  chemistry of, 189-94
  chlorosis and, 199
  disease symptoms, 181-85
  dwarf plant growth and, 195-96
  enzymes and, 200
  epinasty, 196-97
  flowering and, 202-3
  fruiting and, 203
  inhibitors, 186, 187, 188, 194, 196
  leaf expansion, 197-98
  light effects, 203-4
  metabolism and, 199-202
  natural occurrence of, 204
  physiological action of, 194-210
  plants treated with, 206-7
  production of, 185-88
  and roots, 198-99
  seed germination and, 203
  soil moisture and, 184-85
  temperature and, 184
  transport, 198
Gibberellic acid, as vernalin, 407
Glucose
  and auxin delivery, 160
  and gibberellin production, 186, 187
Glutamic acid, 67
Glutamine
  chromatography and, 68, 76
  from urea, 88
  see also Nitrogen metabolism
Glutamyl compounds, 81-84
Glycolic acid oxidase, 444
Grafting, 217-232
  fruiting and, 222
  nutrients and transport, 229-31
  resistance to pests and diseases, 226-29
  root and stem importance, 218-222
  stock/scion incompatibility, 223-26
Grana, 121, 125, 137, 140, 141, 146, 438
Grasses, ecology of, 386
Green plants, mineral nutrition of, 31-59
Griseofulvin, 421
Growing point, 76
Growth
  abnormal, see Gibberellins
  of algae, 309-29
  amino acids and, 76-78
  antibiotic inhibition of, 414-16
  antibiotic stimulation of, 419-21
  chromatographic studies of, 76-78
  climate and, 294-302
  daylength and, 378
  dry weight increase, 199
  of dwarf plants, 195-96
  factor requirements, 402-3
  habits of sugarcane, 276-77, 294-302
  in natural environment, 375-87
  rootstock and scion and, 218-19
  spore, 1
Growth regulators, 161
Guaiacol, 2, 6, 7
Guanidine, 85-86
Guanidyl compounds, 84-90
Gymnosperms, sieve area in, 353, 355

H

Hairs, dye movement and, 356
Hill reaction
  biochemistry of chloroplasts in, 137-49
  inhibitors of, 143-45
  thermal deterioration of, 145-46
Histamine, 76
Hops, 68
Hormone
  cobalt as, 55
  sprays on sugarcane, 301
Humidity
  sap ascent and, 246
  see also Drought resistance
Hydrogen
  and algal culture, 311
  in photosynthesis, 132
Hydroxyacid oxidase, 443-44
Hydroxylamine, 45, 77, 93, 95
Hydroxypipecolic acids, 92-93

I

Imino acids, 90-93
Inarching, 218, 222
Indicator plant, 387
3-Indoleacetaldehyde, as auxin, 159
Indoleacetic acid
  and gibberellins, 205-8
  as inhibitor, 400
  translocation of, 365
  see also Auxin relations in root
Indoleacetic acid oxidase, 446-47
  metals and, 42
3-Indoleacetonitrile, as auxin, 159
3-Indolepyruvic acid, 155, 159
Inheritance, 393
Inhibition
  of Krebs cycle, 84
  and plant distribution, 383-84
Inhibitors
  antibiotics as, 414
  of flower color, 394
  of germination, 375
  of Hill reaction, 143-45
  of laccase, 441
  of phenol oxidase, 437
  of phosphorescence, 128
  and plasmolysis, 336
  respiration, 36
  of rice disease, 186, 187, 188, 194
  of root growth, 153, 158

XUM

of sugar transformation, 289
translocation and, 364, 365
tryptophan, 158
see also Auxin
Injury of cell, 3
Internodes, and rice disease, 194
Interstocks, 221-22
Iodine, absorption of, 53
Ions
accumulation in cells, 4-9
antagonism, 1-3
apparent free space and, 26-27
in cytoplasm, 20
mitochondria and, 28
movement in cell, 11
movement in plant, 37
permeability of, 340-41
and stomatal movement, 259
tonoplast and, 28
uptake of, 12-13, 15-23, 335
see also Root salt absorption
Iron
ascorbic acid deficiency and, 49
chlorosis, tracers in, 31
in leaf, 49
molybdenum and, 57
nickel and, 57
physiological effects of, 48-49
reduction of, 40
translocation of, 40
see also Chlorosis

K

Keto acids
and amino acids, 71-74, 82
in nitrogen metabolism, 65
occurrence of, 74, 76, 82
role of, 74
Krasnovskii reaction, 122
Krebs cycle, 33
inhibition of, 84
mitochondria and, 37

L

Laccase, 440-42
Lamellae, 146
Leaf
cell proteins, 105-6
dye movement in, 357
expansion and gibberellins, 197-98
iron in, 49
osmotic pressure in, 268
phosphorus in, 39
and sap ascent, 250-51
Lecithin, in sugarcane, 280
Legumes, calcium absorption of, 34
Lentil, auxin in root, 156
Lichens, dessication and, 269
Light
actinic, 129
and algal culture, 310, 311-17, 320
amino acids in leaf and, 75
and auxin in root, 161
and carbohydrate metabolism, 261
cobalt movement and, 40
and dormancy breaking, 378
flashing experiments, 117-18, 130, 131
flowering and, 202, 396
fluorescence and, 129
germination and, 378, 397
and gibberellin effect, 198, 203-4
and growth of sugarcane, 294-96
and nitrogen metabolism, 93
phosphorescence and, 128
photoperiod and flowering, 292
photoreaction in germination, 376
and photosynthesis, 145, 264
red, cobalt as, 54
response to, genetic, 395-97
and stomatal movement, 258
and sugar beet, 365
and sugar storage, 275
translocation and, 38
Lignin, 278
Lipides, genetic factor for production, 404
in sugarcane, 278
Lipoxidase, 444-46
Lithium, 53
Lupin, 76
Lysine, 281

M

Magnesium
in mitochondria, 49
phosphorus and, 56
Maize, fungus of, 183
Manganese
and nitrogen metabolism, 93
physiological effects of, 50
Mannitol, cobalt movement and, 40
Marine cell studies, 3-5
Mass flow, in sieve elements, 351
Meristem, auxin and, 169, 170, 172
Metabolism
in cell, 4, 6
glutamyl products and, 83
nitrogen, see Nitrogen metabolism
of sieve elements, 351
solute concentration and, 28
see also Translocation
Metal chelates, role in nutrition, 49
Metallo-flavoproteins, 31, 44
Metals, enzymes and, 42
Microclimate, 376-77
Microsomes, and protein synthesis, 98-99
Middle lamella, 47-48
Millet fungus, 183
Mineral nutrition
of algae, 309-29
ascorbic acid oxidase and, 434
chlorosis, 57-59
elements and, 41-57
genetic factor, 400-1
microelements, 31
salt absorption process, see Root salt absorption
of sugarcane, 281-87
tracers in, 31
translocation, see Translocation
Mint, 75
Mitochondria, 433
calcium and, 36, 44
electrolyte accumulation in, 37
ion uptake and, 28
Krebs cycle and, 37
magnesium and, 49
in nitrogen metabolism, 66
phenol oxidase in, 438
phosphorus and, 43
and protein synthesis, 98-99
respiration in, 139
Mitosis, in auxin-treated root, 172
Molasses, see Sugarcane
Molybdenum
copper and, 56
deficiency of, 51-53
iron and, 57
in nitrogen metabolism, 44, 93, 94
Monocots, cation exchange in, 34
Mosses, dessication and, 269
Mutation
for chlorophyll deficiency, 397-98
see also Genetics

N

Nectaries
sugar in, 361
translocation in, 360-61
Niacin, genetic factor for, 408

XUM

Nickel, 54
Nicotine
  genetic factor for, 408
  synthesis, 51
Nitrate, in marine cells, 4
Nitrate reduction, 93-97
Nitrogen
  auxin and, 160-61
  and carbohydrate metabolism, 261
  and flowering, 294
  metabolism, 44, 47
  reduction, 44-45
  and rice fungus, 188
  in sugarcane, 280-81, 282, 300
  translocation of, 369
Nitrogen fixation, 93-97
  tracers in, 31
Nitrogen-fixing algae, 324-25
Nitrogen metabolism, 65-106
  amino acids in, 73-80
  analytical procedures, 66-70
  carbamyl compounds in, 84-90
  chromatographic procedures, 66-80
  enzymes in, 66
  growth and, 76-78
  guanidyl compounds in, 84-90
  imino acids in, 90-93
  intermediate metabolism, 81-84
  keto acids in, 65, 73-80
  metallo-flavoproteins and, 31
  mitochondria in, 66
  nitrate reduction and, 93-97
  nitrogen compounds in, 70-73
  nitrogen fixation and, 93-97
  port of entry of nitrogen, 96-97
  protein synthesis and, 66, 97-106
  sulfur deficiency and, 75
  urea in, 65-66
Nucleic acid
  in chloroplast, 140
  as ion carrier, 35
  synthesis of, 98
Nucleus
  in chloroplast isolation, 140
  of companion cell, 355
  protein synthesis and, 99
  in sieve elements, 350
Nutrients, in grafting, 229-30
Nutrition, mineral, see Mineral nutrition

O

Oat
  auxin in, 158
  and gibberellins, 198
Ontogenesis, 146
Oranges, 223
Organic acid
  ion absorption and, 34
  in sugarcane, 280
Osmosis, see Cell, permeability of
Osmotic pressure, 1, 5, 339
  apparent free space and, 15
  in cytoplasm, 25-26
  drought and, 267-69
  in mesophyll, 355
  role of, 268
  in root hair, 6
  and stomatal movement, 258
  unequal, 6
  see also Uptake
Osmotic volume
  definition of, 12-14
  determination of, 14-20
  properties of, 20-29
"Outer space," 32, see also Apparent free space
Oxidases, soluble, see Soluble oxidases
Oxidation-reduction, 121-23
  fluorescence and, 123
Oxygen
  in algal culture, 320
  and auxin, 160
  and excited chlorophyll, 121
  flavoproteins and, 442
  as inhibitor of Hill reaction, 144
  and rice fungus, 187
  root absorption and, 31-32
  and translocation, 364
  uptake, 440, 445
  see also Soluble oxidases
Oxytetracycline, see Antibiotics

P

Paper chromatography, see Chromatography
Parasite, toxins from, 416-18
Parenchyma
  carbohydrate transfer in, 354
  functions of, 349
  sieve element in, 352-54
  starch in, 350
Patulin, see Antibiotics
Pea
  auxin in, see Auxin
  chromatographic studies of, 76
  dwarf, 196
  fungus of, 193
Pear, 217
Penicillin, 414
Permeability
  apparent free space and, 11-29
  carrier molecules and, 6
  ion antagonism and, 1-2
  see also Cell
Peroxidase, 350
Pests, resistance to, 226-29
pH
  and algal culture, 325
  and cell permeability, 341-42, 343
  and chloroplast inhibition, 143
  drought resistance and, 272
  effect on sulfur uptake, 39
  and phosphorus absorption, 285
  and rice fungus, 187
  uptake and, 19-20
Phenology, 376-77
Phenol oxidase, 430, 434-40
Phloem
  dyes in, 356-58
  enzymes in, 366
  exudate, 351, 367
  metabolism and movement, 363-70
  movement in, 356-70
  nectaries in, 360-61
  parenchyma cells in, 354-56
  peroxidase in, 350
  phosphorus in, 39
  physiology of, 349-72
  radioactive tracers and, 361-63
  respiration in, 367
  sieve area, 352-54
  sieve element, 350-52
  translocation inhibitors in, 364, 365
  virus movement in, 358-60
Phosphatase, activity in phloem, 354
Phosphate
  and auxin in root, 160
  inhibited translocation of, 365
  root absorption of, 32
Phosphorescence
  of chlorophyll, 119
  in chloroplasts, 128
  in green cells, 128
  inhibitors and, 128
  light and, 128
  temperature and, 128
Phosphorus
  and algal growth, 316
  distribution in tissues, 285
  magnesium and, 56
  physiological effects of, 43
  and plant distribution, 382-83
  root absorption of, 31
  in sugarcane, 280, 284-86, 300
  translocation of, 39, 286-87
  and water in leaf, 286
Phosphorus metabolism, tracers in, 31
Phosphorylation, 66
Photochemistry
  of chlorophyll, 115-34
  flashing light, 131

in vivo, 123-34
spectroscopy, 124-31
steady state fluorescence, 126-27
two-quantum picture, 131-34
Photoperiodism
flowering and, 292
and plant distribution, 377-79
Photosynthesis, 115-34
in algae, 312, 315, 316
antibiotics and, 420
copper and, 50
in drought, 262-64
ecosystem and, 387
electron transfer and, 134
and leaf cell proteins, 105-6
and light, 3
nitrogen fixation and, 45
potassium and, 43
relation to transpiration, 264
stomatal movement and, 263
in sugarcane, 276, 288-89
tracers in, 31
see also Hill reaction
Phototropism, 176-77
Phycocyanin, 127
as light absorber, 146
Physiology
effect of elements, 43-55
genetic factors, 395-408
Phytotoxicity, see Antibiotics
Pigment
fluorescence of, 121
production inhibition, 418-19
Pineapple, auxin in, 158
Pit, see Sieve area
Plankton, 5
Plant
antibiotic effect on, 413-23
distribution, 377-79
growth in natural environment, 375-87
indicator, 387
Plasmalemma, 336, 353
Plasma membrane, 344-45
Plasmodesmata, 352
Plasmolysis, 15
of diatoms, 336
recovery from, 336
in sieve elements, 350
Plastid
constituents, 140
energy transfer in, 127
protein synthesis in, 99
Plum, 217
Pollen, 338
Polymixin, 416
Pore, in plasma membrane, 344
Porphin, 116
Potassium
absorption of, 31, 33, 34
calcium and, 56
movement of, 40
physiological effects of, 43-44
and sugarcane, 283-84, 300
translocation of, 286-87
Proline, 90
Protein
and absorption carriers, 34
algae as source of, 327
and auxin binding, 162, 174
in chloroplasts, 140
and nitrogen metabolism, 66
occurrence of amino acids in, 70-73
photosynthesis in leaf and, 105-6
in plasma membrane, 345
synthesis, 97-106
see also Chlorosis
Protoplasm, 337
electrical resistance of, 2
Protoplast
dessication and, 269
ions and, 28
permeability and, 336
sieve-element, 350-52
Pseudoplasmolysis, 270

Q

Quantum conversion, 148-49

R

Radiation ecology, 388
Raspberries, 51
Reproduction, zinc and, 50
Reproductive parts, elements in, 43
Respiration
in algae, 315
"anion," 36
ascorbic acid oxidase and, 428, 432
auxin and, 160
calcium and, 36
cyanide and, 36
and drought resistance, 264-66
dye movement and, 357
ecosystem, 387
flavoproteins and, 443
and gibberellins, 201
in grafted stock, 230
and laccase, 442
metabolite concentration and, 28
phenol oxidase and, 438
potassium and, 43
protein cycle in, 104
"salt," 35-37, 38
silicon and, 55
stimulants, 36
in sugarcane, 290-91
and translocation, 367
Ribonucleoproteins, as absorption carriers, 33
Rice
and algae, 325
fungus disease, see Gibberellins
Ring-grafting, 221, 222
Ripeness, and flowering, 294
Ripening, of sugarcane, 299-301
Root
adventitious, 364
anchorage in grafting, 228
auxin and external factors, 160-61
auxin occurrence in, 154-58
auxin relations, 153-77
auxin transport in, 161-62
boron deficiency and, 51
decapitated sections and auxin, 171-72
exudate of sugarcane, 283
geotropism in, 176
and gibberellins, 198-99
growth and external auxin, 164-73
growth inhibition, 172-73, 414-16
growth of, 154
intact, and auxin, 164-68
isolated, and auxin, 168-71
mineral nutrition and, 31-37
osmotic pressure in, 268
phototropism in, 176-77
and stem, 218-22
strontium in, 41
swelling and auxin, 172-73
tip production of auxin, 158-60
see also Root salt absorption
Root pressure
and sap ascent, 250
of sugarcane, 297
Root salt absorption, 31-37
aeration, 31-32
bioelectric potentials, 35
carrier concept, 32-34
exchange adsorption, 32-34
movement from root, 37
"salt respiration," 35-37
Rootstock
effect on fruiting, 222-23
growth and, 218-19
morphology, 220
scion relationships, 217-31
stem and root importance, 218-22
vigour, 220
Rubber, 402
Rubidium, absorption of, 53

S

"Salt respiration," 35-37, 38

Salts
permeability of, 340-41
and rice fungus, 186
and stomatal movement, 259
see also Ions
Sap ascent, 237-53
cohesion theory, 238-49
vital process theory, 250-51
Scion
and growth, 218-19
roots, 218
and rootstock incompatibility, 223-26
stem, 219-21
and stock relations, 217-31
variety of, 220
vigour, 220
Seed
amino acids in, 71
chromatographic studies of, 76
genetics of germination, 399
germination and light, 396
germination rhythms, 375
and gibberellins, 201, 203
premature germination of, 399
respiration in, 265-66
in rice fields, 325
of sugarcane, 296
Seedling growth, inhibition of, 414
Selenium, 54
Senescence, 146
Sieve area, strands in, 352-54
Sieve-element, 350-52
Silicon, respiration and, 55
Smudge, 401
Sodium
absorption of, 34
calcium and, 56
physiological effects of, 53
potassium and, 56
Soil
antibiotic production in, 413
climate near, 377
ecological distribution and, 381-83
and flowering, 293-94
metal chelates and, 49
and rice disease, 184-85
root absorption and, 31
serpentine, 382
water condensation in, 380
Soluble oxidases, 427-47
amine oxidase, 443
ascorbic acid, 428-34
hydroxyacid oxidase, 443
indoleacetic acid oxidase, 446-47
laccase, 440-42
lipoxidase, 444-46
phenol oxidase, 434-40
Sorghum, 183
Soybean, 197
Spectroscopy, 124-31
chlorophyll reactions and, 115-20
transient, 127-31
Spore
growth, 1
of rice disease, 183
Spray
damage, 228
and flowering, 294
Starch
in parenchyma, 350
and stomatal movement, 258
Stem
elongation and fungus, 194-95
in grafting, 218-22
see also Sap ascent
Stomata
movement of, 258-61
of sugarcane, 297
Strontium, 41
absorption of, 53
Sugar
accumulation of, 43
apparent free space and, 24
in arctic-alpine species, 379
boron and, 41
in cell, 11
in chloroplast isolation, 139, 140
cobalt and, 40, 55
deterioration of, 301-2
effect on tracer movement, 37
equilibrium, 145
and habitat moisture, 262
movement of, 365
in nectaries, 360-61
and plasmolysis, 270
and stomatal movement, 258
storage in sugarcane, 299-301
in sugarcane, 284
transformations, 289-90
translocation of, 51, 362, 363
transport of, 365-68
uptake, nonosmotic, 335
Sugar beet, 365-66
chlorine and, 54
sodium and, 53
Sugarcane plant, 275-302
carbohydrate metabolism, 288-92
climate and, 294-302
composition, 277-81
enzymes in, 290, 291
flowering of, 292-94
fungus, see Gibberellins
growth habits, 276-77, 294-302
mineral nutrition, 281-87
photosynthesis in, 288-89
post-harvest deterioration, 301-2
respiration in, 290-91
ripening, 299-301
varietal yield, 276
water and, 296-99
Sulfite, 40
Sulfur
calcium and, 46
pH and, 45-46
translocation of, 39-40, 45-46
Sunflower, 197
Synergism, see Grafting

T

Tannin, 141
as Hill reaction inhibitors, 143-44
in sugarcane, 278
Taxonomy, 78-80
Tea, 197
Temperature
and algal culture, 310, 312, 316-17, 319, 323
and alpine plants, 377
cobalt movement and, 40
and dormancy breaking, 378
dye movement and, 357
and flowering, 293
freezing and chloroplasts, 142
and germination, 375
and growth of sugarcane, 294-96
and phosphorescence, 128
and phosphorus absorption, 285
respiration and, 364
and rice fungus disease, 184, 187
sap ascent and, 246, 252
thermal deterioration of cells, 145-46
translocation and, 38, 363-64
Terramycin, see Antibiotics
Thiourea, 119, 121, 339
Tissue culture, antibiotics and, 419
Tobacco
chromatographic studies of, 75
and gibberellins, 197
Tobacco mosaic, 359
Tomato
carbohydrate movement, 363
genes for color, 397
and gibberellin, 197
phosphorus in, 39
Tonoplast, 337
and dye permeation, 343
ions and, 28
Toxicity, 1
Tracheids, 242
Transamination, 65
Translocation
of antibiotics, 421-22

XUM

of calcium, 38-39
of cobalt, 40
of iron, 40
metabolism and, 363
in mineral nutrition, 37-41
of phosphorus, 39
of potassium, 40
region of, 38
in sugarcane, 291
of sulfur, 39-40
see also Phloem
Transpiration
boron and, 51
ion movement and, 37
osmotic pressure and, 267
relation to photosynthesis, 264
stomata and, 263
see also Sap ascent
Transport, in grafted stock, 230-31
Tree
ascent of sap, 237-53
calcium in, 38
photoperiodicity in, 378
see also Rootstock
Triiodobenzoic acid, 161
Triphenylene, 119
Tryptophan
and gibberellins, 201
as root growth inhibitor, 158
Tulip, 78
Turgescence, see Drought resistance

U

Uptake
initial, 12-13
methods and, 15-20
see also Apparent free space
Urea
cycle, 86-87
as Hill reaction inhibitor, 147
in nitrogen metabolism, 65-66, 84-85
permeability of, 339
in sugarcane, 287
utilization of, 87-88

V

Vacuole
auxin in, 155, 157
dessication and, 269-70
electrolytes in, 4, 6
osmotic pressure and, 25-26
uptake and, 23
Vanadium, 54
Vascular bundle, movement in, 362
Vessels, 242, 246, 248
Virus
as Hill reaction inhibitors, 144
movement in phloem, 358-60
protein synthesis in, 98, 103
and stock and scion relations, 217, 222, 224
of sugarcane, 276
synthesis of, 103
Vitamins
algae as source of, 327
cobalt in, 55
gene for vitamin B, 408
in sugarcane, 278-79

W

Water
column continuity, 241-44
in conducting elements, 244-48
and flowering, 294
growth and, 3
movement in cell, 5,6
nonosmotic uptake, 335
permeability of, 339, 340
photolysis, 148-49
and photosynthesis, 288
and plant ecology, 379-81
soil moisture and rice fungus, 184-85
and sugarcane plant, 296-300
tensile strength, 238-41
transport of, 237, 335
see also Drought resistance, Permeability, Transpiration
Wax, 279
Weber's law, 2
Wheat
apparent free space in root, 15, 24
fungus, 183
Winter frost, resistance to 227-28
Wool, gibberellin fungus and, 186

X

Xanthine oxidase, 42
Xylem
movement in, 361-62
phosphorus in, 39
see also Sap ascent
Xylem sap, 79

Y

Yeast
and gibberellins, 201
permeability of, 341-42
potassium uptake carrier in, 35

Z

Zinc, 50